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Choline Supplementation Mitigates Trace, but not Delay, Eyeblick Conditioning Deficits in Rats Exposed to Alcohol During Development

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Abstract

Children exposed to alcohol prenatally suffer from a range of physical, neuropathological and behavioral alterations, referred to as Fetal Alcohol Spectrum Disorders (FASD). Both the cerebellum and hippocampus are affected by alcohol exposure during development, which may contribute to behavioral and cognitive deficits observed in children with FASD. Despite the known neuropathology associated with prenatal alcohol exposure, many pregnant women continue to drink (heavy drinkers, in particular), creating a need to identify effective treatments for their children who are adversely affected by alcohol. We previously reported that choline supplementation can mitigate alcohol's effects on cognitive development, specifically on tasks which depend on the functional integrity of the hippocampus. The present study examined whether choline supplementation could differentially mitigate ethanol's effects on trace eyeblink classical conditioning (a hippocampal-dependent task) and delay eyeblink classical conditioning (a cerebellar-dependent task). Long-Evans rats were exposed to 5.25 g/kg/day alcohol via gastric intubation from postnatal days (PD) 4-9, a period of brain development equivalent to late gestation in humans. A sham-intubated control group was included. From PD 10-30, subjects received subcutaneous injections of 100 mg/kg choline chloride or vehicle. Beginning on PD 32-34, subjects were trained on either delay or trace eyeblink conditioning. Performance of subjects exposed to alcohol was significantly impaired on both tasks, as indicated by significant reductions in percentage and amplitude of conditioned eyeblink responses, an effect that was attenuated by choline supplementation on the trace, but not delay conditioning task. Indeed, ethanol-exposed subjects treated with choline performed at control levels on the trace eyeblink conditioning task. There were no significant main or interactive effects of sex. These data indicate that choline supplementation can significantly reduce the severity of trace eyeblink conditioning deficits associated with early alcohol exposure, even when administered after the alcohol insult is complete. These findings have important implications for the treatment of fetal alcohol spectrum disorders.

Keywords

fetal alcohol; treatment; ethanol; hippocampus; learning

Introduction

Children with fetal alcohol spectrum disorders (FASD) suffer from a range of behavioral problems that include attention deficits, learning impairments, motor dysfunction, emotional disturbances and altered social behavior (Kodituwakku, 2007; NIAAA, 2000; Riley and McGee, 2005). Despite the known adverse effects of prenatal alcohol exposure, women continue to drink alcohol during pregnancy and it is estimated that as many as 1 in 100 births may be affected by alcohol exposure during gestation (Sampson et al., 1997). Thus, an important challenge is to identify methods to reduce the severity of these disorders and improve outcome among individuals with FASD.

Although prenatal alcohol exposure disrupts development of many CNS regions, some of the observed alcohol-induced behavioral alterations may be related to hippocampal dysfunction (Riley et al., 1986). Indeed, animal studies indicate that developmental alcohol exposure leads to structural and functional changes in the hippocampus that correspond to deficits in spatial and trial specific memory, behaviors that rely on the functional integrity of the hippocampus (see Berman and Hannigan, 2000 for review). Similarly, clinical studies of individuals with FASD indicate that hippocampal volumes may be reduced, although in proportion to overall reduced brain size (Archibald et al., 2001), with some hemispheric asymmetries (Riikonen et al., 1999). Moreover, hippocampal dysfunction with concomitant deficits in spatial learning have been reported among individuals with FASD (Hamilton et al., 2003). Protection against alcohol-induced hippocampal damage may prevent some of these behavioral alterations.

Animal studies have demonstrated that perinatal choline supplementation can alter hippocampal development. Choline is recognized as an essential nutrient by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences (Food and Nutrition Board, 1998). Perinatal choline supplementation alters morphology of cholinergic cells of the basal forebrain (Loy et al., 1991; Williams et al., 1998), as well as hippocampal pyramidal cells (Li et al., 2004). Perinatal choline supplementation can also lead to enhanced efficiency of cholinergic functioning in the hippocampus and cortex (Blusztajn et al., 1998; Cermak et al., 1999; Cermak et al., 1998; Coutcher et al., 1992; Meck et al., 1989; Montoya et al., 2000), and lowers the threshold of hippocampal long-term potentiation (LTP) (Jones et al., 1999; Pyapali et al., 1998), a mechanism of plasticity believed to underlie some learning and memory. Finally, prenatal choline supplementation also enhances mitogen-activated protein kinase (MAPK) and cAMP response-activated binding protein (CREB) activation in hippocampal slices (Mellott et al., 2004). These CNS changes may be evident months after termination of choline treatment.

Consistent with choline's effects on the brain, perinatal choline supplementation also leads to long-lasting cognitive enhancement. Rats that receive choline supplementation during prenatal and/or early postnatal development exhibit enhanced memory and reduced proactive interference on tasks of spatial learning like the radial arm maze and Morris water maze (Brandner, 2002; Meck et al., 1988; Meck et al., 1989; Meck and Williams, 1997b; Meck and Williams, 1999; Meck and Williams, 2003; Tees and Mohammadi, 1999). Perinatal choline supplementation also enhances temporal memory (Cheng et al., 2006; Meck and Williams, 1997a; Meck and Williams, 1997c) and attentional processing (Meck and Williams, 1997b). As with the changes in brain functioning, choline-induced cognitive enhancement is long-lasting, evident even months after choline administration and during periods of development equivalent to old age, suggesting that choline may reduce age-related memory decline as well (Glenn et al., 2008; Meck et al., 2007).

We have been examining the effectiveness of choline supplementation in rats exposed to alcohol during development and found that it significantly mitigates alcohol-related hyperactivity (Thomas et al., 2004a; Thomas et al., 2007), as well as deficits in spatial learning (Ryan et al., 2008; Thomas et al., 2007), working memory (Thomas et al., in press; Thomas et al., 2000), reversal learning (Thomas et al., 2004a), and trace fear conditioning (Wagner and Hunt, 2006). Such mitigation can occur even when choline supplementation occurs after alcohol exposure is complete (Thomas et al., 2007), indicating that choline can be effective in reducing some of ethanol's teratogenic effects, even after the ethanol-related injury has occurred. The present study investigates whether choline can mitigate ethanol's effects on different forms of eyeblink conditioning. Eyeblink conditioning is a form of associative learning that is commonly studied in mammalian neuroscience because stimuli can be discretely delivered, performance and learning measures can be easily separated, its neural basis has been mapped out in detail, and it has cross-species applications – even in humans (Goodlett et al., 2000; Green, 2004; Steinmetz, 2000).

Delay eyeblink classical conditioning (ECC), during which the onset of a conditioned stimulus (CS) precedes and overlaps with the onset of an unconditioned stimulus (US), depends on the functional integrity of a specific brain stem-cerebellar circuit (Lavond, Kim, & Thompson, 1993; Thompson, 1986). In contrast, trace ECC is a procedure that is mediated by indirect cerebellar-hippocampal interactions. Unlike delay ECC, trace ECC requires that the offset of the CS precedes the onset of the US, with a period during which no stimulus is present (i.e., the trace period). The hippocampus, as well as the medial prefrontal cortex, is critical for maintaining the memory of the CS (the memory trace) as the organism is required to bridge the association between CS and US events (McLaughlin et al., 2002; Moyer et al., 1990; Weible et al., 2000; Weiss, Bouwmeester, Power, & Disterhoft, 1999). Indeed, rats with damage to the hippocampus cannot successfully perform this task but can perform the delay task (Ivkovich and Stanton, 2001), particularly when the delay task involves shorter inter-stimulus intervals (Beylin et al., 2001). Moreover, the pattern of hippocampal neural activation, particularly in area CA1, is greater during trace ECC than delay ECC (Green and Arenos, 2007), likely because of presynaptic nicotinic acetylcholine receptor involvement in facilitating glutamatergic release during this task (Rodriguez-Moreno et al., 2006). Since both forms of ECC have a firm neurobiological basis, clearly depending on either the integrity of the brain stem-cerebellar circuit or the integrity of the hippocampus, these tasks are ideal for examining choline's role in minimizing the deleterious effects of developmental alcohol exposure, and whether the alcohol-damaged hippocampus is more responsive than an equally vulnerable cerebellum to choline under more specific task demands.

The present study examined whether choline supplementation administered after 3rd trimester equivalent alcohol exposure and during a time equivalent to early postnatal development in humans could reduce the severity of eyeblink conditioning deficits. In this study, we exposed rats to alcohol during the 3rd trimester equivalent brain growth spurt (postnatal days (PD) 4-9) and then administered choline daily from PD 10-30, which would be equivalent to early postnatal development in humans. After choline treatment was completed, subjects were tested on either the delay or trace ECC procedure.

Materials and Methods

Subjects and Treatment

Generation of litters—Long-Evans female rats were bred overnight with male breeders in the vivarium of the Behavioral Neuroscience lab at East Carolina University. This vivarium was maintained on a 12 hr light/12 hr dark cycle. The following day, sperm smears were examined and any females with positive smears (i.e., detection of sperm during estrus)

were regarded as being at gestational day (GD) 0. Dams were housed individually and received access to food and water *ad libitum*. Body weights were measured each day to track for proper growth during their pregnancies. On the day of birth (GD 22), the pups were regarded as being on PD 0. On PD 3, each litter was pseudorandomly culled to 8 pups (4 males, 4 females whenever possible) and paw-marked with non-toxic ink.

Neonatal and perinatal treatments—On PD 4, the pups were randomly selected to receive one of four within-litter neonatal treatments: (1) ethanol (EtOH), (2) ethanol + choline (EtOH + C), (3) sham intubation (SI), and (4) sham intubation + choline (SI + C). Only one rat of each sex (1 male, 1 female) within each litter was randomly placed into any of these treatment groups. From PD 4-9, EtOH and EtOH + C pups were weighed and intragastrically intubated with 5.25 g/kg ethyl alcohol (EtOH) in milk formula, delivered over two feedings (2 hr apart). The total EtOH volume in milk was 11.9% (v/v) and the milk formula was derived from that used by West, Hamre, and Pierce (1984). Two additional milk-only intubations were given to EtOH subjects (each 2 hr apart) to supplement the pups' lack of suckling to the mother while under the influence of EtOH. Each intubation volume was 0.0278 ml/g per feeding and the total intubation volume across the four feedings **each day** was 0.1112 ml/g. The SI and SI + C groups were also weighed and received the intubation procedure during PD 4-9, but without milk or EtOH; sham intubations served to control for any potential intubation stress that the EtOH and EtOH + C rats received throughout this neonatal period. This milk formula and administration method incorporating SI pups have been used extensively elsewhere (see Green et al., 2002b; Brown et al., 2007; Tran et al., 2005, 2007). There were no unintubated control rats (i.e., unhandled suckle controls) used in this study, as it has been found in many previous reports using this neonatal alcohol exposure model that there are no differences in ECC between sham intubated and unintubated controls (Green et al., 2002b; Tran et al., 2005; Tran et al., 2007). Therefore, the addition of this group would have required an increased production of litters that was reasoned to be scientifically unnecessary.

From PD 10-30, rats designated for choline administration (EtOH + C, SI + C) were weighed and received daily subcutaneous injections of choline chloride (100 mg choline/kg/day; 25 ml of 70% choline chloride [DuCoa, Verona, MO] was added to 0.85% saline for a concentration of 18.8 mg choline chloride/ml). The other two groups of rats (EtOH, SI) were also weighed but received saline injections (0.85%, s.c.) during this perinatal period. This is the same treatment regimen used in our previous studies (Thomas et al., 2007; Ryan et al., 2008). On PD 21, all rats were weaned from their mothers and housed in same-sex pairs with food and water *ad libitum*.

All prenatal, neonatal, and perinatal procedures were approved by the East Carolina University Animal Care and Use Committee (ACUC) and were conducted in strict accordance with the guidelines that were set forth.

Blood Ethanol Concentrations (BECs)

On PD 6, tail blood samples were collected from all pups 1.5 hr after the second EtOH or sham intubation. The samples were analyzed for EtOH content using an enzymatic procedure first described by Dudek and Abbott (1984) that has been used previously (Tran et al., 2000; Tran and Kelly, 2003). Briefly, 10 microliters of blood from each pup were collected in microcentrifuge tubes containing 0.53N perchloric acid, neutralized with 0.30M potassium carbonate, and then spun at 14,000 RPM for 15 min. Samples were stored at -20°C until time of assay (Tran and Kelly, 2003).

Eyelid Surgery

On the day of surgery ($PD\ 32 \pm 2$), rats were separated from their littermates and housed individually for the remainder of the study. Isoflurane® was used as the surgical anesthetic. During surgery, a small incision was first made to reveal the cranium. A fabricated electromyographic (EMG) “headstage” containing two stainless steel recording wires (3T, Medwire Corp., Mt. Vernon, NY) and a stainless steel ground wire (10T) was then fitted atop the cranium. The two recording wires (one positive, one negative) were inserted through the left eyelid, slightly dorsal to the eyelid muscle (*orbicularis oculi*). The ground wire was positioned towards the lambda suture line and concealed subcutaneously. A bipolar stimulating electrode (Plastics One Inc., Wallingford, CT) used for delivery of the shock US, was placed immediately caudal to the left eye. This was achieved by separating the skin from the temporal muscle to create a small “pocket” for the contacts of the bipolar electrode to be situated. The plug end of the bipolar electrode was situated atop the cranium at the midline and aligned with the EMG headstage. The incision area containing the EMG headstage and bipolar plug was then sealed using Ortho-Jet dental cement (Lang Dental, Wheeling, IL). After surgery, the rats were given Buprenex analgesia (.03 mg/ml sc; corrected for body weight) and monitored for full recovery before being replaced in the vivarium. All aseptic surgical and postoperative procedures were followed according to guidelines set for by the ECU ACUC.

Apparatus

The testing apparatus consisted of a modified operant conditioning box containing a house light (20 W) and a fan (55-60 dB). Each box was housed inside a 25 (W) $\times$ 16 (D) $\times$ 23.5 (H) in chamber (Med Associates, St. Albans, VT) that was fitted with polyurethane acoustical foam (McMaster-Carr, Atlanta, GA) to help attenuate environmental noise. The tone CS was produced by a piezo horn tweeter situated directly above the operant box, and was calibrated to 80 dB (at 2.8 kHz) using a digital sound level meter prior to the study. The shock US was produced by a stimulus isolator (Model A365 World Precision Instruments, Sarasota, FL) that was confirmed to deliver 2.0 mA of current.

The animal’s EMG headstage was plugged into a 5-channel commutator (Plastics One, Inc., Roanoke, VA), which allowed for seamless retention of electrical signals if a rat moves about the operant box. The commutator was connected directly to the eyeblink system (JSA Designs, Raleigh, NC) that included an EMG amplifier that amplified the raw signal (5,000 $\times$), an EMG Integrator that rectified the raw signal, and a stimulus controller device which controlled the delivery of the CS and US. The eyeblink system was connected to an IBM-compatible pc installed with proprietary ECC software (JSA Designs) that recorded the EMG activity in either 2 ms (delay ECC) or 3.5 ms (trace ECC) bins during each trial epoch. This software also controlled the delivery of the CS and US via the Stimulus Controller unit.

Eyeblink Classical Conditioning (ECC)

Delay ECC—During each trial, a tone CS (80 dB, 2.8 kHz) was presented first and following a delay of 280 ms, a shock US (60 Hz, 2 mA) was delivered. The shock US remained on for 100 ms, co-terminating with the tone CS. There were 90 total CS-US trials in one session; the average inter-trial interval was 30 sec (18 - 42 sec). However, on every 10th trial, the tone CS was presented by itself to test for learning of the CR. In total, there were 100 trials per session. Rats received two sessions per day (each session 5 hr apart) over three consecutive days, thus receiving a total of six sessions.

Trace ECC—Like delay ECC, rats underwent TECC within 48 hr after eyelid surgery. The stimulus parameters, number of sessions, and number of training days were the same as those for delay ECC, with the exception of the CS-US presentation. Unlike delay ECC, the

tone CS was presented first and remained on by itself for 380 ms. The tone then terminated and after a 500-ms delay, the shock US was delivered and remained on for 100 ms. This 500-ms time window represented the interval during which the animal is required to depend on a memory trace to form an association between the tone CS and the shock US, allowing it to emit well-timed eyeblink conditioned responses after the offset of the CS but immediately prior to the delayed onset of the US.

Data Collection and Statistical Analysis

The data were pre-screened for acceptable and unacceptable trials within each session using criteria developed by Skelton (1988). All relevant measures (below) associated with “acceptable” trials were averaged within session. The screening process was carried out with assistance from proprietary data analysis software (JSA Designs, Raleigh, NC), which divided each trial epoch into four discrete EMG sampling periods that were matched between both ECC procedures (see Figure 1): (1) a 280-ms pre-CS period that allowed measurement of baseline activity before tone CS onset, (2) a startle response (SR) period during the first 80 ms after CS onset (EMG activity related exclusively to a non-associative reaction), (3) a 200-ms adaptive CR period that allowed for measuring well-timed CRs prior to shock US onset, and (4) a UR period which measured EMG activity that occurred from the onset of the US to the end of the trial (140 ms). Any EMG activity that exceeded the pre-CS baseline mean by at least 0.4V (2 standard deviations) was registered by the software as an SR, CR, and/or UR during their respective sampling periods. The session means for each of these measures were obtained over 90 possible CS-US trials or 10 possible CS-alone trials. Preliminary analysis of the proportion of eliminated trials using a EtOH (EtOH vs. Sham) $\times$ choline (Choline vs. Vehicle) $\times$ Sex $\times$ ECC Training (Delay vs. Trace) mixed ANOVA with session as the repeated-measures variable, showed no significant main effects or interactions, confirming that the eliminated trials did not influence variability in the SR, CR, and UR measures noted below.

The relevant measures that were analyzed for each response type included percentage and amplitude of SRs, adaptive CRs, and URs, as has been conducted in previous reports using the PD 4-9 binge alcohol exposure model (e.g., Brown et al., 2007; Stanton & Goodlett, 1998; Tran et al., 2005, 2007). The reason for analyzing the adaptive CR is that it represents a well-timed eyeblink response just prior to (i.e., 200 ms before) US onset, which is mediated by the cerebellar cortex in delay ECC (Ivkovich, Paczkowski, & Stanton, 2000; Perrett, Ruiz, & Mauk, 1993) or hippocampus in trace ECC (Ivkovich & Stanton, 2001; Moyer et al., 1990). This method of comparing the same adaptive CR window between different ECC procedures has been utilized in many different studies (e.g., see Claflin et al., 2005; Ivkovich & Stanton, 2001). Furthermore, adaptive CRs that were expressed (as measured by frequency and amplitude) during CS-alone trials were analyzed. CS-alone trials were examined because they represent test trials for learning CS-US associations, as exhibited by CR percentage and CR amplitude.

Data for the SR, CR, and UR measures were analyzed using ANOVAs with EtOH (EtOH vs. Sham), choline (Choline vs. Vehicle), and sex as between-subjects factors, separately for trace ECC and delay ECC. In all cases, the repeated-measures factor was session (6). Follow-up ANOVAs were conducted when appropriate and simple effects tests were performed on significant interactions. All statistical analyses were conducted using a minimum alpha level of .05.

Results

Growth

Body weights (*g*) were analyzed from PD 4-14, 15, 21 and 30 to determine whether perinatal choline or neonatal ethanol treatment produced growth differences (see Table 1). Body weights from PD 4-14 were analyzed using a 2 (sex) $\times$ 2 (neonatal EtOH) $\times$ 2 (perinatal choline) $\times$ 2 (ECC training) $\times$ 11 (day) mixed analysis of variance (ANOVA), with day as the repeated variable. Ethanol exposure slowed somatic growth. As expected, there was a significant effect of postnatal day, $F(10, 660) = 1,553$, $p < .001$, confirming normal growth across days, and a significant EtOH $\times$ Day interaction, $F(10, 660) = 4.8$, $p < .001$. The EtOH $\times$ Day interaction was subjected to simple effects test, which revealed that EtOH pups lagged behind SI rats on PD 8-14 (p 's $< .05$). There were no other significant interactions with the day variable. Importantly, there were no main or interactive effects of choline on body growth.

The body weights that were collected on PD 15, 21 and 30 were subjected to separate between-subjects ANOVAs with sex, neonatal EtOH, perinatal choline, and ECC training as the factors. PD 15 analysis showed a significant main effect of EtOH, $F(1, 66) = 4.1$, $p < .05$, as ethanol-exposed pups were slightly, but significantly, smaller than sham-intubated pups (see Table 1). No other significant main or interactive effects were found. The smaller body weights in EtOH rats diminished on PD 21, where the analysis revealed a significant main effect of ECC training, $F(1, 66) = 6.9$, $p < .05$, and no other significant effects. Rats that were assigned to delay ECC were smaller in body weight compared to those that were assigned to trace ECC (47.5 ± 1.0 g vs. 51.2 ± 1.0 g). By PD 30, the only significant differences in body weight were related to sex, $F(1, 66) = 10.3$, $p < .01$, which was expected by this age; males weighed significantly more than females (106.8 ± 1.5 g vs. 100.3 ± 1.4 g). Most importantly, the minor growth differences that were observed during the early neonatal treatment period (from PD 8-14) between ethanol-treated pups and sham-intubated pups diminished rapidly as the subjects grew into periadolescence.

Blood Ethanol Concentrations

Mean $\pm$ *SEM* blood ethanol concentrations were 346 ± 22 mg/dl for EtOH rats, and 331 ± 18 mg/dl for EtOH + C rats that received delay ECC. For trace ECC rats, these concentrations were 326 ± 12 mg/dl (EtOH) and 316 ± 17 mg/dl (EtOH+C). Two samples from trace ECC rats could not be analyzed due to collection and/or storage error. However, the results for this group still were within expected values for EtOH treatment at the 5.25 g/kg dose. A 2 (EtOH / EtOH + C) $\times$ 2 (delay vs. trace ECC) between-subjects ANOVA confirmed that the BECs were not significantly different between treatment groups or testing condition. Importantly, these blood alcohol levels are clinically relevant, achievable with binge-like exposure (May et al., 2008; Reynaud et al., 2001).

Acquisition: Percentage of adaptive CRs

Data for 82 rats were acquired for all analyses. Some data loss occurred as a result of mortality during intubations ($n = 2$ EtOH+C trace; $n = 1$ SI trace; $n = 1$ SI+C trace; $n = 2$ EtOH delay; $n = 2$ SI delay) and screened ECC data that did meet criteria for acceptability as noted previously ($n = 1$ EtOH+C trace; $n = 1$ SI+C trace; $n = 1$ SI delay). The final number of rats for each condition is shown in Figure 2.

Ethanol exposure during the 3rd trimester equivalent significantly impaired acquisition of trace eyeblink conditioning and this was mitigated with choline supplementation. The percentage of adaptive CRs during CS-alone trials was analyzed using a 2 (Sex) $\times$ 2 (Choline) $\times$ 2 (EtOH) $\times$ 6 (Session) mixed ANOVA, with session as the repeated variable.

There were no main or interactive effects of sex on any of the outcome measures. The analysis confirmed a significant main effect of EtOH, $F(1, 35) = 10.50, p < .01$, as well as a main effect of perinatal choline treatment $F(1, 35) = 16.48, p < .001$ and a significant EtOH $\times$ Choline interaction, $F(1, 35) = 9.18, p < .01$. Follow-up analyses revealed that rats which received neonatal EtOH treatment without choline were impaired in acquiring adaptive CRs compared to controls and to EtOH-treated rats that received choline supplementation. Simple effects analyses also confirmed that neither the controls (SI vs. SI + C) nor the choline-treated groups (EtOH + C vs. SI + C) differed significantly (p 's $> .50$). There was also an EtOH $\times$ Session interaction $F(1, 175) = 2.92, p < .05$, as performance did not improve as much over session in the ethanol-exposed subjects compared to all other groups. Simple effects tests conducted on the EtOH $\times$ Session interaction confirmed that rats which received neonatal EtOH treatment performed significantly worse than SI rats during sessions 3, 4, and 6 (p 's $< .05$), an effect driven by the ethanol subjects not treated with choline. Thus, the performance of ethanol-exposed subjects not treated with choline was significantly impaired compared to all other groups, including the ethanol-exposed subjects treated with choline (see Figure 2a).

Choline supplementation, however, did not mitigate the ethanol-induced impairments in delay eyeblink conditioning. A significant main effect of EtOH was found, $F(1, 39) = 20.49, p < .001$, but no significant effects of choline or a EtOH $\times$ Choline interaction. Rats that received EtOH during PD 4-9 were significantly impaired in acquiring CRs compared to those that received sham intubations ($47.9\% \pm 3.44$ vs. $70.23\% \pm 3.54$, respectively) but chronic choline exposure alone during PD 10-30 did not enhance learning compared to saline vehicle ($60.74\% \pm 3.28$ vs., $57.4\% \pm 3.69$), indicating that choline did not mitigate ethanol-induced behavioral impairment in this cerebellar-based task (Figure 2a). As expected, there was a main effect of session $F(5, 195) = 51.28, p < .001$, as the CRs developed over training.

Acquisition: Amplitude of adaptive CRs

Overall, the ANOVA of CR amplitude revealed similar results to those observed for percentage of CRs. During the trace ECC testing, the CR amplitude was severely diminished in rats that received ethanol during PD 4-9. The ANOVA revealed a main effect of EtOH $F(1, 35) = 5.22, p < .05$, and choline $F(1, 35) = 4.21, p < .05$, but no significant EtOH $\times$ Choline interaction ($p = .08$). Although the EtOH $\times$ Choline interaction failed to reach significance, the choline effect was driven by the EtOH subjects that received choline (Figure 2b). In fact, there were no significant differences in CR amplitude among the EtOH + C and control groups. The CR amplitude exhibited by sham-intubated controls was higher than that exhibited by EtOH rats ($2.64 \pm .34$ V vs. $1.64 \pm .33$ V) and choline administration produced similar findings compared to vehicle exposure ($2.59 \pm .36$ V vs. $1.69 \pm .32$ V). As expected there was a significant effect due to session, $F(5, 175) = 25.43, p < .001$, and a significant EtOH $\times$ Session interaction, $F(15, 175) = 2.98, p < .05$, as EtOH subjects not treated with choline illustrated no increase in response amplitude over sessions.

During the delay ECC condition, there was only a significant main effect of EtOH, $F(1, 35) = 13.33, p < .001$, but no significant main or interactive effects of choline. Sham-intubated controls exhibited higher CR amplitude across six sessions of training compared to EtOH rats ($4.17 \pm .41$ V vs. $2.07 \pm .40$ V), producing a main effect of session $F(5, 195) = 48.72, p < .001$, as well as an EtOH $\times$ Session interaction, $F(5, 195) = 5.51, p < .005$, due to slower increases in CR amplitude over training sessions among the EtOH subjects. Overall, these findings are consistent with those in the CR percentage measure, indicating that choline did not mitigate ethanol-induced behavioral impairment in this task which critically involves the cerebellum (Figure 2b). There are, notably, differences in adaptive CR amplitude between testing conditions (trace compared to delay ECC), which may be reflective of overall diminishment

in CR strength exhibited by all groups as a result of the difficulty of trace compared to delay ECC.

Non-Associative and Sensory Measures

Startle responses—The percentage and amplitude of non-associative SRs during CS-alone trials were both analyzed using global mixed ANOVAs. Both analyses showed no significant main effects or significant interactions. The mean percentages and mean amplitudes were well below levels that would impact the corresponding learning (i.e., CR) measures. The overall mean percentage of SRs (pooled across sessions) were: (1) trace (EtOH = 6.21 ± 2.37 , EtOH+C = 8.38 ± 3.52 , SI = 10.96 ± 3.22 , SI+C = 9.83 ± 3.45) and (2) delay (EtOH = 10.05 ± 3.21 , EtOH+C = 10.30 ± 3.04 , SI = 7.03 ± 2.11 , SI+C = 10.50 ± 2.34). The overall mean SR amplitudes were: (1) trace (EtOH = 0.24 ± 0.16 , EtOH+C = 0.40 ± 0.19 , SI = 0.79 ± 0.39 , SI+C = 0.70 ± 0.35) and (2) delay (EtOH = 0.49 ± 0.25 , EtOH+C = 0.61 ± 0.25 , SI = 0.43 ± 0.20 , SI+C = 0.44 ± 0.18). These findings confirm that any differences in initial reaction to the tone CS were unrelated to the aforementioned differences amongst groups in learning the contingency between the CS and US.

Unconditioned responses—The percentage and amplitude of URs during paired CS-US trials (90 per session) were both analyzed in a similar manner as the SR measures. Like the SR analyses, both analyses showed no significant main effects or interactions. The overall mean percentage of URs were: (1) trace (EtOH = 98.89 ± 0.62 , EtOH+C = 97.30 ± 2.31 , SI = 98.23 ± 1.03 , SI+C = 99.44 ± 0.36) and (2) delay (EtOH = 94.83 ± 2.04 , EtOH+C = 98.08 ± 1.17 , SI = 96.78 ± 2.22 , SI+C = 96.01 ± 2.33). The overall mean UR amplitudes were: (1) trace (EtOH = 4.53 ± 0.56 , EtOH+C = 5.31 ± 0.89 , SI = 3.95 ± 0.72 , SI+C = 4.79 ± 0.80) and (2) delay (EtOH = 4.24 ± 0.61 , EtOH+C = 4.94 ± 0.66 , SI = 4.20 ± 0.66 , SI+C = 4.78 ± 0.75). These findings confirm that any differences in sensory responding to the shock US were nearly nonexistent, and such differences did not influence the lack of CR acquisition in group EtOH (in either delay or trace conditions) compared to all other groups.

Discussion

This study is the first to demonstrate that perinatal choline supplementation can significantly mitigate ethanol's effects on trace eyeblink conditioning. These findings are consistent with previous research showing that perinatal choline supplementation reduces the severity of deficits in trace fear conditioning (Wagner and Hunt, 2006), as well as deficits on other cognitive tasks (Thomas et al., 2007; Thomas et al., 2004a; Thomas et al., 2000), associated with developmental alcohol exposure. Overall, the findings indicate that choline may serve as an effective treatment for ethanol's damaging effects on cognitive ability.

Similar to previous findings, ethanol exposure during the 3rd trimester equivalent brain growth spurt disrupted normal acquisition of eyeblink conditioning. Alcohol exposure during this period of development disrupts eyeblink conditioning in both delay (Green et al., 2002a; Green et al., 2002b; Stanton and Goodlett, 1998; Tran et al., 2005; Tran et al., 2007) and trace conditions (Tran and Goodlett, 2003; Tran and Goodlett, 2004), presumably related to alcohol-induced damage to the cerebellum and hippocampus, respectively. It is likely that the deficits in trace eyeblink conditioning observed are related to hippocampal dysfunction, as alcohol exposure during the 3rd trimester leads to both structural and functional alterations in the hippocampus, including cell loss in the CA1 field, as well as alterations in synaptic connections, electrophysiological responses, and neurochemistry (Berman and Hannigan, 2000).

While it is possible that cerebellar damage contributes to the ethanol-related deficits in trace conditioning, choline did not attenuate ethanol-related deficits on the delay conditioning

paradigm, which depends on the functional integrity of the cerebellum. These findings are consistent with previous research showing that choline supplementation during this postnatal period (PD 4-30) reduces the severity of reversal learning (Thomas et al., 2004a), spatial learning (Thomas et al., 2007), and overactivity in an open field (Thomas et al., 2007; Thomas et al., 2004a), as well as trace fear conditioning (Wagner and Hunt, 2006), all behaviors that rely on the functional integrity of the hippocampus. In contrast, choline supplementation during this period of development did not improve alcohol-related deficits in parallel bar motor coordination (Thomas et al., 2004b), suggesting that administration of choline on PD 10-30 specifically influences functioning of the hippocampus and/or cortex, but not areas such as the cerebellum.

The neural circuitry of classical eyeblink conditioning is among the most well understood learning circuits. Delay ECC depends on the functional integrity of the the cerebellar cortex, specifically long-term depression at parallel fiber-Purkinje cell synapses (Kishimoto et al., 2001; Thompson & Krupa, 1994). Trace eyeblink conditioning is spared in humans with cerebellar damage, particularly when the trace interval is long (1000 ms) but not short (400 ms) (Gerwig et al., 2008). Similarly, trace ECC, but not delay ECC, is spared in Purkinje cell degeneration (pcd) mice (Brown et al., 2010). That being said, some cerebellar circuitry may contribute to trace conditioning, particularly in the topography of the CR. Single-unit activity of cerebellar interpositus neurons is present regardless of whether rats produce CRs in delay or trace conditions, but the intensity of the activation varies according to different time signatures (early training vs. later training), possibly representing encoding of CS duration (Green & Arenos, 2007). In guinea pigs, bicuculline microinjections into the cerebellar interpositus nucleus failed to abolish previously acquired trace CRs, but the CR topographies (peak latencies were shortened and peak amplitudes diminished) were altered (Hu et al., 2010). Thus, although it is possible that choline has a beneficial effect in recruiting cerebellar activity to modulate trace ECC, given lack of a choline effect on delay conditioning and the low number of muscarinic receptors in the anterior interpositus nucleus (Beitz et al., 1984; Lavond, 2002; Woolf & Butcher, 1989), it is more likely that the responsivity of the hippocampus to choline treatment in this study contributed to the spared trace learning deficits in alcohol-exposed rats.

In addition, other cortical areas may also modulate acquisition, retention, and storage of trace CRs. Bilateral lesions of the caudal medial prefrontal cortex (cmPFC) in rabbits severely impair the acquisition of the CR without influencing extinction, whereas lesions of the rostral medial prefrontal cortex (rmPFC) retards the extinction of the CR without influencing its acquisition (Kronforst-Collins & Disterhoft, 1998; Weible et al., 2000). The patterns of activation of neurons in the cmPFC recorded in vivo suggest an attentional role for this structure early in training (Weible et al., 2003) as they are evident during the earliest CS-US presentations, prior to evidence of learning. Lesion studies suggest that the retrosplenial cortex does not mediate acquisition or extinction of the CR, but provides the long-term retention of critical task information (Berger, Weikart, Bassett, & Orr, 1986; Sears & Steinmetz, 1990) as it serves as an indirect output pathway from the hippocampal formation to the cerebellum via the pontine nuclei (Weiss & Disterhoft, 1996). It is possible that these cortical areas are also responsive to choline administration to mediate acquisition of trace ECC when challenged with neonatal alcohol exposure.

Although little is known of the effects of early postnatal choline supplementation on brain function, prenatal choline supplementation in non-alcohol exposed rats has been shown to lead to a variety of changes in the hippocampus (Li et al., 2004). For example, choline availability during development can influence cell division, migration and differentiation of hippocampal cells (Albright et al., 1999; Albright et al., 2005; Albright et al., 1998), leading to long-lasting increases in cell size and basal dendritic arborization in CA1 pyramidal

neurons (Li et al., 2004). But more importantly, perinatal choline supplementation leads to long-lasting changes in hippocampal plasticity that continue throughout the lifespan. Prenatal choline supplementation enhances N-methyl-D-aspartate (NMDA) receptor neurotransmission (Montoya and Swartzwelder, 2000), excitability of CA1 pyramidal cells (Li et al., 2004), and long-term potentiation (LTP) (Jones et al., 1999; Pyapali et al., 1998), as well as a number of intracellular signals, including phospholipase D activity (Holler et al., 1996), mitogen-activated protein kinase (MAPK) and cAMP response-activated binding protein (CREB) activation (Mellott et al., 2004). Prenatal choline may exert some of these effects by increasing various neurotrophic factors (Sandstrom et al., 2002; Mellott et al., 2007; Glenn et al., 2007). Prenatal choline supplementation even increases adult hippocampal neurogenesis (Glenn et al., 2007; Glenn et al., 2008). Although the effects of choline supplementation on hippocampal structure and function following administration specifically on PD 10-30 have yet to be elucidated, given that developmental alcohol exposure impairs hippocampal plasticity (see Berman and Hannigan, 2000), and that trace eyeblink conditioning depends on hippocampal plasticity, including NMDA receptor function (Kishimoto et al., 2006), LTP (Christian and Thompson, 2003) and neurogenesis (Shors et al., 2001), choline supplementation likely attenuates ethanol-related deficits by targeting such processes.

In the present study, choline did not significantly alter behavior of controls. Prenatal choline supplementation has been shown to improve cognitive performance on a range of tasks (Meck et al., 1988; Meck and Williams, 1997a; Meck and Williams, 1997b; Meck and Williams, 2003; McCann et al., 2006). However, in most of those studies subjects received diets supplemented with choline during the prenatal period (predominantly GD 11-17), whereas the present study administered choline during a later developmental period. Indeed, postnatal choline supplementation is less effective in otherwise typically developing rats compared to prenatal choline supplementation (Meck et al., 1989). Certainly the consequences of choline supplementation will depend on the developmental timing of administration, and collectively, the data to date suggest that choline may influence brain development of subjects exposed to alcohol during development, even during developmental periods when it does not significantly affect performance of control subjects, suggesting that choline may be more effective in a subject who sustains brain damage, compared to intact subjects. In fact, choline supplementation following kainic-acid induced status epilepticus on PD 35 protects against hippocampal-based behavioral alterations, suggesting that postnatal choline may have effects on hippocampal function in the compromised brain that are not evident in control subjects (Holmes et al., 2002).

The mechanisms of choline's actions are not yet known. Choline supplementation during development may lead to metabolic imprinting, influencing cholinergic functioning throughout the lifespan by altering availability of choline from phosphatidylcholine stores (Meck and Williams, 2003). Indeed, prenatal choline supplementation leads to greater efficiency in cholinergic functioning both in the hippocampus and cortex, effects that are evident even in adulthood, months after choline supplementation (Blusztajn et al., 1998; Cermak et al., 1999; Cermak et al., 1998; Coutcher et al., 1992; Meck et al., 1989; Montoya et al., 2000). Acetylcholine release in the hippocampus is known to modulate learning, including trace eyeblink conditioning (Disterhoft and Matthew Oh, 2003; Fontan-Lozano et al., 2005) and alcohol exposure during the 3rd trimester equivalent alters hippocampal cholinergic functioning (Kelly et al., 1989). Thus, early choline loading may lead to enhanced cholinergic functioning in the hippocampus and this might contribute to enhancements in trace eyeblink conditioning.

However, choline may also alter brain organization and function via other mechanisms as well. Choline can exert neurotrophic effects by directly activating α -7 nicotinic receptors

(Albuquerque et al., 1997; Mike et al., 2000). Choline is also a precursor to cell membrane constituents, phosphatidylcholine and sphingomyelin, as well as signaling factors, platelet activating factor and sphingosylphosphorycholine (Zeisel, 2006; Zeisel and Niculescu, 2006). In addition, choline acts as a source of biologically labile methyl groups which can act as epigenetic factors, influencing gene expression (Niculescu et al., 2006; Zeisel and Niculescu, 2006). Thus, choline may alter brain and behavioral development via a number of sites of action. It is likely that choline's specific actions will depend on the developmental timing of administration and mechanisms that may be at work during prenatal development may not be the same as those that occur later in development, during PD 10-30. Examination of the neural changes associated with the behavioral changes observed in the present study will hopefully help to elucidate choline's targets.

Importantly, choline supplementation occurred after the cessation of alcohol exposure, during a period of development that would be equivalent to early postnatal life in humans, suggesting that choline could be administered postnatally and still be effective. Moreover, behavioral testing occurred after choline treatment was complete, so the effects observed in the present study were not due to the acute action of choline, but to longer lasting changes in CNS functioning. These findings suggest that choline could be administered during early postnatal development among humans exposed to alcohol prenatally and still be effective in reducing some fetal alcohol effects. These results have important implications for the treatment of the adverse effects of early alcohol exposure, suggesting that dietary interventions may prove effective among individuals with FASD.

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Figure 1.

Comparison of delay and trace eyeblink classical conditioning procedures. (a) In the delay procedure, a tone conditioned stimulus (CS) is presented and remains on for 380 ms. At 280 ms into the CS onset, a shock unconditioned stimulus (US) is delivered to the rat. Both stimuli remain on for 100 ms and co-terminate. Eyeblink responses that are emitted after the shock US is delivered are regarded as unconditioned responses (URs), while eyeblink responses that are emitted in anticipation of the shock US (200-ms adaptive CR period) are considered conditioned responses (CRs). With some amount of exposure to the CS-US pairings, rats with an intact cerebellum exhibit normal acquisition (i.e., CR) curves. (b) In the trace procedure, the tone CS is also delivered before the onset of the shock US, however, it terminates prior to the US (at 380 ms), thereby leaving a 500-ms “trace” period in which the rat is required to bridge the association between the CS and the US. Rats with an intact hippocampus are also expected to emit CRs in this task, but the timing of the CRs in relation

to onset of the US will shift to a well-timed “adaptive” window (200 ms prior to US onset) with adequate training. In both panels, the stippled horizontal line represents the threshold value of 0.4 V above the pre-CS baseline mean, which any given peak must surpass in order to be registered as a blink response. Startle responses (SRs) are nonassociative responses that may occur within the first 80 ms of a trial epoch and are not considered CRs.

Figure 2.

Acquisition of eyeblink conditioned responses (CRs) in delay and trace eyeblink classical conditioning (ECC). The data represent mean responses during CS-alone trials in terms of (a) percentage of adaptive CRs ($\pm$ SEM) and (b) adaptive CR amplitude ($\pm$ SEM) across six sessions (S) of training. Periadolescent rats that were treated with ethanol (EtOH) during postnatal days (PD) 4-9 that were subsequently given saline (EtOH) or choline (EtOH+C) supplementation from PD 10-30, were significantly impaired in acquiring adaptive CRs in delay ECC compared to sham-intubated controls (SI, SI+C). However, ethanol-exposed rats that received choline supplementation (EtOH+C) were spared from the deleterious effects of ethanol in trace ECC, compared to ethanol-exposed rats that did not receive choline (EtOH) with respect to CR percentage. The performance of EtOH+C rats were not significantly different from SI and SI+C controls.

Table 1
Mean Postnatal Body Weights

Training × Group	PD 4	PD 8 ^a	PD 14 ^a	PD 15 ^b	PD 21 ^c	PD 30 ^d
<u>Delay ECC</u>						
EtOH (<i>n</i> = 10)	9.6 ± 0.5	15.0 ± 1.0	28.6 ± 1.3	30.6 ± 1.3	46.9 ± 1.4	100.6 ± 2.8
EtOH+C (<i>n</i> = 12)	9.6 ± 0.5	15.2 ± 1.1	28.7 ± 1.5	30.8 ± 1.5	46.9 ± 2.3	103.3 ± 4.5
SI (<i>n</i> = 9)	9.5 ± 0.5	16.9 ± 1.2	30.6 ± 1.4	32.5 ± 1.4	48.5 ± 2.4	101.8 ± 3.3
SI+C (<i>n</i> = 12)	9.2 ± 0.4	16.0 ± 0.9	30.3 ± 0.9	32.7 ± 0.9	48.2 ± 1.7	102.9 ± 3.8
<u>Trace ECC</u>						
EtOH (<i>n</i> = 12)	10.4 ± 0.3	16.1 ± 0.5	29.5 ± 0.9	31.7 ± 1.0	50.7 ± 1.6	103.5 ± 1.8
EtOH+C (<i>n</i> = 8)	10.4 ± 0.3	16.6 ± 0.5	30.6 ± 1.0	32.3 ± 0.7	50.1 ± 0.9	103.5 ± 1.7
SI (<i>n</i> = 10)	10.5 ± 0.5	17.9 ± 0.5	32.1 ± 1.0	34.5 ± 1.1	52.8 ± 1.7	107.4 ± 2.6
SI+C (<i>n</i> = 9)	9.8 ± 0.3	17.1 ± 0.7	31.2 ± 1.1	33.6 ± 1.2	50.8 ± 2.3	106.3 ± 2.9

Note. The values represent mean weight (g ± *SEM*). Body weights were obtained daily from PD 4-30 and the data have been truncated above for the purposes of display.

^aEtOH groups (EtOH and EtOH+C) weighed less than both SI groups during PD 8-14; *p* < .05.

^bEtOH groups weighed less than SI groups; *p* = .048.

^cDECC groups weighed less than TECC groups; *p* = .011.

^dMales weighed more than females; *p* = .002.