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CHOLINE PARTIALLY PREVENTS THE IMPACT OF ETHANOL ON THE LIPID RAFT DEPENDENT FUNCTIONS OF L1 CELL ADHESION MOLECULE

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Abstract

Background—Fetal Alcohol Spectrum Disorder, the leading known cause of mental retardation, is caused by alcohol exposure during pregnancy. One mechanism of ethanol teratogenicity is the disruption of the function of L1 cell adhesion molecule (L1). These functions include enhancement of neurite outgrowth, trafficking through lipid rafts, and signal transduction. Recent data have shown that choline supplementation of rat pups reduces the effects of ethanol on neurobehavior. We sought to determine if choline could prevent the effect of ethanol on L1 function using a simple experimental system.

Methods—Cerebellar granule neurons (CGN) from postnatal day 6 rat pups were cultured with and without supplemental choline, and the effects on L1 signaling, lipid raft distribution and neurite outgrowth were measured in the presence or absence of ethanol.

Results—Choline significantly reduced the effect of ethanol on L1 signaling, the distribution of L1 in lipid rafts and L1 mediated neurite outgrowth. However, choline supplemented ethanol exposed cultures remained significantly different than controls.

Conclusions—Choline pretreatment of CGN significantly reduces the disruption of L1 function by ethanol, but does not completely return L1 function to baseline. This experimental system will enable discovery of the mechanism of the neuroprotective effect of choline.

Keywords

L1 cell adhesion molecule; ethanol; lipid raft; choline; fetal alcohol spectrum disorder

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) is a pattern of mental and physical defects that develop in a fetus as a consequence of ethanol consumption during pregnancy. In the United States, up to 30% of pregnant women report using alcohol at some point during pregnancy

(Havens et al., 2009). Fetal Alcohol Syndrome (FAS) which is at the severe end of FASD is hallmarked by postnatal growth restriction, craniofacial abnormalities and central nervous system dysfunction (Stratton et al., 1996). At least one third to one half of children born to chronic alcoholic mothers show some signs of FASD. FASD prevalence is estimated to be 1% of all live births in Seattle WA (Sampson et al., 1997) and up to 20% in parts of South Africa (May et al., 2013).

L1 cell adhesion molecule (L1) has been implicated in the pathogenesis of FAS as a result of the extensive overlap of neuroanatomic features between FAS and the disorders caused by mutations in the gene for L1 (Charness et al., 1994; Ramanathan et al., 1996). L1 is a member of the Ig superfamily and is a transmembrane glycolipoprotein with a tyrosine containing cytoplasmic domain, and an extracellular domain with 6 IgG-like repeats and 5 fibronectin-like repeats (Maness and Schachner, 2007). The transmembrane structure of L1 allows it to activate signal transduction pathways in the neuron following homophilic binding. L1 plays a role in axon growth and fasciculation, neuronal migration, synaptic plasticity and regeneration (Maness and Schachner, 2007). L1 mediates neurite outgrowth through a downstream signaling cascade consisting of activation of pp60^{src} and ERK1/2, dephosphorylation of tyrosine 1176 in the cytoplasmic domain of L1, and phosphorylation of other tyrosines in the cytoplasmic domain of L1 (Schmid et al., 2000; Schaefer et al., 1999; Schaefer et al., 2002; Tuvia et al., 1997; Yeane et al., 2009). This cascade is inhibited by ethanol (Yeane et al., 2009; Bearer et al., 1999; Littner et al., 2013; Tang et al., 2006; Watanabe et al., 2004). The neurite outgrowth of cerebellar granule neurons (CGN) grown on a substrate of the extracellular domain of L1 is effectively suppressed by ethanol at pharmacologic concentrations (Bearer et al., 1999).

Increasing evidence indicate the role of lipid rafts in the mechanism of ethanol toxicity. Lipid rafts are fluctuating nanoscale assemblies of sphingomyelin, cholesterol, GM1 gangliosides and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking (Tsui-Pierchala et al., 2002). L1-mediated neurite outgrowth is dependent on L1-lipid raft interactions. Neurite outgrowth on L1 is inhibited in the presence of lipid raft disrupting agents: 1) methyl- β -cyclodextrin, a cholesterol chelator (Tang et al., 2011; Nakai and Kamiguchi, 2002), 2) lovastatin, an inhibitor of cholesterol synthesis (Nakai and Kamiguchi, 2002); and 3) fumonisins B1, an inhibitor of sphingolipid synthesis (Nakai and Kamiguchi, 2002). Ethanol exposure increases the proportion of L1 present in lipid rafts, but not N-cadherin, both *in vitro* and *in vivo* (Littner et al., 2013; Tang et al., 2011) suggesting an impact of ethanol on protein trafficking through lipid rafts.

Choline is an essential nutrient in humans, and is important as a methyl group donor and as a precursor to acetylcholine and membrane phospholipids, including sphingomyelin. Choline supplementation reduces the severity of neurobehavioral outcomes in rat pups previously or concurrently treated with ethanol (Thomas and Tran, 2012; Thomas et al., 2010; Thomas et al., 2009; Ryan et al., 2008; Thomas et al., 2007; Thomas et al., 2004; Thomas et al., 2000). Choline is a critical component of both phosphatidylcholine and sphingomyelin in lipid rafts. In the present study, we sought to determine if supplementation of CGN with choline prior to ethanol exposure would prevent the effects of ethanol on L1 functions dependent on lipid rafts.

MATERIALS AND METHODS

Reagents and Antibodies

Betaine, acetylcholine chloride, choline chloride, and phosphocholine, and phosphatidylcholine were obtained from Sigma-Aldrich (St. Louis, MO). Water (LC/MS grade), acetonitrile (LC/MS grade), acetic acid (LC/MS grade), ethanol (HPLC grade), glacial acetic acid (HPLC grade), and formic acid (LC/MS grade) were from Fisher Scientific (Pittsburg, PA). The chimeric protein consisting of the extracellular domain of L1 and the Fc domain of IgG (L1-Fc) was purchased from R&D System (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated cholera toxin B subunits (CTx-B) were purchased from Sigma. Goat polyclonal anti-neural cell adhesion molecule L1 (polyclonal antibody to the cytoplasmic domain of L1 (anti-L1CD) and mouse monoclonal anti-c-Src (anti-pp60src) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to the dephosphorylated Y1176 of L1 (74-5H7) was obtained from Covance (Berkeley, CA). Polyclonal antibodies to phospho-Src family (Tyr416), phospho-p44/42 mitogen activated protein kinase (MAPK) (Thr202/Tyr204)(pERK1/2), p44/42 MAPK (total ERK) and monoclonal antibody to phosphotyrosine (P-Tyr-100) were obtained from Cell Signaling (Beverly, MA). Mouse monoclonal anti-beta III tubulin was from Invitrogen. Alexa Fluoro 488 dye conjugated goat anti-mouse was obtained from Molecular Probes (Eugene, OR). All secondary antibodies for immunoblot analysis were from Jackson Immuno Research Laboratories (West Grove, PA). Mouse monoclonal antibody to rat L1 (ASCS4) developed by P.H. Patterson was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

METHODS

Cell cultures

Primary cultures of rat CGN were prepared from 6 day old Sprague-Dawley rat cerebella as previously described (Bearer et al., 1999). All procedures employing experimental rats were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland Baltimore and in accordance with the guidelines for animal care established by the National Institutes of Health. Briefly, rat pups were rapidly decapitated, their cerebella were isolated and immersed in ice cold phosphate buffered saline (PBS). The cells were dissociated in cold buffer that contains 0.05% trypsin-EDTA and collected by centrifugation. Viability of these dissociated cells was assessed with trypan blue and was >90%.

Neurite length

Cells were plated on poly L-lysine (PLL) coated coverslips at a density of 2×10^4 cells/ml and cultured in serum free-defined media. Serum free-defined media (SF) consisted of Neurobasal Media (Gibco, Rockville, MD), with the following additions: 2% B27 supplement (Gibco), 20 mM L-glutamine, 6 g/l glucose, 20 mM HEPES, pH 7.2, and penicillin/streptomycin. 0.2 µg/ml L1-Fc and/or choline were added to the media immediately after plating. Ethanol, 25 mM, was added 2 h after plating. After 24 h, cells

were fixed using 4% paraformaldehyde for 30 min at room temperature and labeled fluorescently for beta III tubulin. A masked investigator identified eligible neurites for measurement using an a priori design. Eligible neurites were the longest neurite of a CGN that meets the following criteria: neurite is longer than the cell soma, the neuron does not touch other neurons, and the neurite does not touch other neurites. Cells were viewed on a Zeiss Observer Z1 fluorescence inverted microscope (Carl Zeiss, Oberkochen, Germany). Images were captured using Axiovision camera software (Carl Zeiss), and neurite length was measured using Image J software (National Institutes of Health, Bethesda, MD) (Bearer et al., 1999; Tang et al., 2011).

Sample preparation for choline determination

CGN were incubated overnight in DMEM, 20 mM HEPES, 10% fetal bovine serum (FBS) and penicillin/streptomycin. Some cultures were supplemented with 40 μ M choline. Half the cultures were treated with 25 mM ethanol for 1 hour prior to washing. Cells were washed three times with PBS and lysates made using RIPA buffer from Sigma. 100 μ L of cell lysate solution was mixed with 10 μ L of internal standard (100 μ M of acetylcholine). 500 μ L of acetonitrile was added for protein precipitation. The solution was mixed for 30 sec and centrifuged for 10 minutes at 10K rpm. 500 μ L of supernatant was transferred to another tube and dried under nitrogen. The sample was re-suspended in H₂O/Acetonitrile (1:1 v/v with 0.1% acetic acid). The sample was further diluted 50 fold for analysis.

Choline analysis via high performance liquid chromatography electrospray ionization-tandem mass spectrometry (HPLC ESI-MS/MS) using a method adapted from Zeisel and coworkers (Koc et al, 2002). HPLC ESI-MS/MS was carried out on an Ultimate 3000 RS Liquid Chromatogram system coupled to a TSQ Quantum Ultra Tandem Quadrupole Mass Spectrometer (Thermo Fisher, San Jose, CA). The HPLC separation was performed on an Agilent Zorbax RX-SIL column (2.1 $\times$ 150 mm, 5.1 μ m) operated at 30 °C. An acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid (800/127/68/3/2, v/v; solvent A) and acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid (500/500/85/27/18, v/v; solvent B) gradient was used. The gradient was 0% B (0.4 mL/min) for 3 min, to 40% B (0.4 mL/min) in 7 min, to 45% B (0.3 mL/min) in 4 min, to 60% B (0.3 mL/min) in 4 min, to 100% B (0.5 mL/min) in 1 min, maintained at 100% B (0.5 mL/min) for 8 min, to 0% B (0.4 mL/min) in 1 min, and maintained at 0% B for 2 min. The injection volume was 10 μ L and HPLC auto-sampler was maintained at 10 °C. Positive ions were created using a spray voltage of 3 kV, capillary temperature of 350°C, and tube lens of 35 V. Positively charged ions from choline (Cho) and acetylcholine (AcCho) were monitored in selected reaction monitoring (SRM) mode for mass spectrometry detection. The transitions used for detection were as follows: Cho m/z 104 $\rightarrow$ 60 and AcCho m/z 146 $\rightarrow$ 87. Data collection, analysis, and quantitation were performed by XCalibur V 2.1 (Thermo Fisher).

Calibration Standards

Stock solutions of Cho and AcCho were prepared in water:acetonitrile (1:1 v/v) with 0.1 % formic acid. Cho calibration standards ranging from 0.1 μ M to 50 μ M spiked with 10 μ M AcCho (internal standard) were prepared in RIPA buffer (H₂O:ACN (1:1 v/v) with 0.1 % formic acid. Quality control (QC) samples at 3 concentration levels (1, 10, and 50 μ M) were

also prepared. Calibration standards and QC samples were prepared fresh daily and analyzed in replicate analyses before and after biological samples.

Signal transduction

CGN were plated on PLL in DMEM, 20 mM HEPES, 10% FBS and penicillin/streptomycin (complete media, CM), and treated with or without additional choline. Cells were grown overnight, then placed in medium without serum for 2 h. After 2 h, ethanol was added for 1 h, then L1 was clustered by the addition of a cross-linked antibody as previously described (Yeaney et al., 2009; Tang et al., 2006). To form cross-linked ASCS4 antibody complexes, purified ASCS4 was mixed with goat anti-mouse IgG (H+L)(1:2.5 g/g) for 1 h at 4°C. The complexes were added to cultures for a final concentration of 30 µg/mL of media. Mouse IgG 30 µg/mL final concentration was added as control (Schmid et al., 2000; Yeaney et al., 2009; Tang et al., 2006). 10 min after addition of the complexes, the tissue culture dishes were placed on ice, the media was removed, and the CGN were washed with ice-cold Hank's Buffered Saline Solution (HBSS). Cells were lysed on ice in lysis buffer containing: 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM Na-vanadate, 10 mM NaF, 2 µM aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, 1 µg/mL pepstatin A, 10 µg/mL turkey trypsin inhibitor, phosphatase inhibitor cocktail I (Sigma), and phosphate inhibitor cocktail II (Sigma). The cell lysates were cleared by centrifugation for 15 min at 16,200 g at 4°C. For immunoprecipitation with anti-L1CD or anti-cSrc, the cell extract supernatants were transferred to clean microfuge tubes and pre-cleared with 1 µg rabbit IgG or mouse IgG for 1 h, then with 30 µL Protein A/G-agarose for 1 h, followed by low speed centrifugation. Supernatants were collected and mixed with 2 µg of indicated antibody. After 1 h incubation, 30 µL of protein A/G-agarose was added and lysates were incubated overnight. The protein A/G-agarose was collected by centrifugation at 1000 g for 4 min and washed three times with ice-cold PBS. Proteins were eluted and denatured in Laemmli sample buffer containing 5% β-mercaptoethanol. For immunoblots, cell lysates were denatured in Laemmli sample buffer containing 5% β-mercaptoethanol. Proteins were separated by SDS-PAGE (12% gel) and transferred to a hydrated polyvinylidene fluoride membrane. The membrane was blocked in 5% fat free milk and 0.1% Tween 20, incubated with primary antibodies (74-5H7, Tyr416, pERK1/2), washed, then secondary antibodies (HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG), and reactive bands visualized using Fluorchem HD2 imaging system (Alpha Innotech, Santa Clara, CA). Blots were stripped and reprobed with anti-L1CD, anti-pp60src or anti-total ERK1/2 with appropriate secondary antibodies and reactive bands visualized. The blots were analyzed in a semiquantitative manner using Image J (NIH, Bethesda) software to measure the density of bands on the blots. The 74-5H7 band densities were normalized for the band densities of L1CD, the band densities of Tyr416 were normalized for the band densities of pp60src, and the band densities of pERK1/2 were normalized for the band densities of total ERK1/2. Relative densitometric units were determined by calculating the ratio of the normalized band densities to that of the control.

Lipid raft isolation and determination of L1 distribution

Lipid rafts from whole cells were prepared as follows: CGN were cultured overnight in CM with or without supplemental choline. 3 h prior to harvest, the cells were serum starved. 1 h

prior to harvest, 25 mM ethanol was added to some cultures. Prior to harvest, all equipment was cooled to 4°C. The media was removed, and cells were washed with cold HBSS. CGN were scraped, and lysed in low detergent buffer consisting of 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 10 mM Na-vanadate, 2 μM aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μg/mL pepstatin A, 10 μg/mL turkey trypsin inhibitor, 100 pM cypermethrin, phosphatase inhibitor cocktail I (Sigma), and phosphate inhibitor cocktail II (Sigma). The lysates were centrifuged for 30 min at 13,000 g at 4°C. After centrifugation, 2 mL of the lysate supernatant was mixed with an equal volume of 80% sucrose solution and then overlaid by a sucrose density gradient with 32% and 5% layers. The gradient was centrifuged at 180,000 g, for 24 h at 4°C. Sequential 1 mL fractions were drawn off the top of the gradient. An aliquot from each fraction was analyzed by immunoblot analysis for both transferrin receptor and GM1 ganglioside. All GM1 ganglioside containing fractions identified by reactivity with CTX-B were combined (lipid raft pool (LR)). All remaining fractions were combined (non-lipid raft pool (non-LR)). Proteins from an equivolume from each pool were precipitated using chloroform/methanol and resuspended in Laemmli sample buffer for immunoblot. Immunoblots were quantified for L1 in each pool, and the % of L1 in the lipid raft pool was calculated as $100 \times \text{L1 in LR} / (\text{L1 in LR} + (\text{L1 in non-LR}))$.

Statistical Analysis

Means, standard deviations and t-test p-values were calculated using Microsoft Excel software.

RESULTS

To determine the concentration of choline to be used in the following experiments, the effect of different concentrations on neurite length was screened in a single cell preparation. As we have shown previously, addition of L1 to CGN plated on PLL caused a 2 fold increase neurite length, and the addition of 25 mM ethanol completely inhibited this increase (Figure 1). There was an abrupt increase of neurite length between 20 and 40 μM choline added to the media of CGN grown in the presence of L1 and ethanol (Figure 1). Increasing the concentration of choline above 40 μM had no further effect on neurite length. Addition of 40 μM choline to 1) CGN grown on PLL alone, 2) PLL with 25 mM ethanol, or 3) PLL with L1 added to the media had no significant effect on neurite length. Based on this screen, 40 μM choline was the concentration of choline investigated in these experiments.

To determine if ethanol altered choline content of CGN, we measured intracellular choline content in CGN with or without 25 mM ethanol, and in CGN supplemented with choline for 24 h with or without 25 mM ethanol (Figure 2). In unsupplemented CGN, ethanol caused a small but significant decrease in intracellular choline. Choline supplementation increased the choline content of CGN by 10 fold (Figure 2). Ethanol had no effect on choline content of CGN treated with choline.

We have previously shown that ethanol inhibits L1 stimulated tyrosine phosphorylation of its cytoplasmic domain, L1 activation of ERK1/2 and p60src, and L1 stimulated dephosphorylation of Y1176 (Yeaney et al., 2009; Tang et al., 2006). As shown in Figure 3,

choline supplementation partially prevented the inhibition by ethanol of phosphorylation of the tyrosines of L1. In addition, choline treatment alone did not increase tyrosine phosphorylation of L1. In Figure 4, choline pretreatment partially prevented ethanol inhibition of L1 activation of ERK1/2. In both cases, choline addition in the absence of L1 stimulation had no effect on L1 tyrosine phosphorylation or ERK1/2 activation, implying that the effect of choline was specifically on inhibition of the effect of ethanol.

In further experiments shown in Figure 5 and 6, choline pretreatment also prevented the ethanol inhibition of L1 activation of p60src (Figure 5) and L1 stimulated dephosphorylation of tyrosine 1176 in the cytoplasmic domain of L1 recognized by the monoclonal antibody 74-5H7 (Figure 6).

We have previously shown that ethanol alters the distribution of L1 in lipid rafts both *in vitro* (Tang et al., 2011) and *in vivo* (Littner et al., 2013). As shown in Figure 7, choline addition alone did not alter L1 distribution in lipid rafts. Ethanol increased the proportion of L1 in lipid rafts, and choline partially prevented this effect.

To determine the effect of 40 μ M choline, CGN were plated on PLL, then L1, ethanol, and/or choline were added to the media (Figure 8). Neurite length was measured after 24 h of incubation (Bearer et al., 1999). 40 μ M choline had no significant effect on neurite length of CGN grown on PLL alone or PLL + L1. L1 addition to CGN markedly increased the mean neurite length compared to CGN grown on PLL alone. 25 mM ethanol markedly reduced the neurite length of CGN grown on L1. Choline supplementation reduced the effect of ethanol in that mean neurite length was significantly longer than in the presence of L1 and ethanol alone (51.5 ± 8.5 versus 30.6 ± 1.2 microns, $p < 0.05$). However, the protective effect was not complete in that the length of neurites with L1, ethanol and choline was significantly shorter than L1 alone (51.5 ± 8.5 versus 67.8 ± 6.8 microns, $p < 0.05$).

DISCUSSION

In this study using a simple experimental system, we show that choline pretreatment increases the cellular content of choline, ethanol has a minimal effect on choline uptake, and choline reduces the effects of ethanol.

Our laboratory has focused on L1 cell adhesion molecule, a transmembrane protein critical for the development of the brain (Fransen et al., 1998; Fransen et al., 1996; Fransen et al., 1995). L1 functions through signaling cascades and protein trafficking, most notably trafficking through lipid rafts (Tang et al., 2011; Nakai and Kamiguchi, 2002). We have shown the ethanol interferes with this trafficking both *in vivo* and *in vitro*, and disrupts L1 signaling (Fig. 2 – 8, (Yeaney et al., 2009; Littner et al., 2013; Tang et al., 2006; Tang et al., 2011; Tang et al., 2011)). Recent evidence from several laboratories supports the important role of lipid rafts in ethanol toxicity (Sebastian and Nagy, 2005; Szabo et al., 2007; Blanco et al., 2008). Lipid rafts are enriched in sphingolipids such as sphingomyelin, which is typically elevated by 50% compared to the plasma membrane (Pike, 2009). Choline is a critical component of both phosphatidylcholine and sphingomyelin in lipid rafts. We sought to examine in this study, whether choline supplementation would reduce ethanol disruption

of L1 signaling, trafficking and promotion of neurite outgrowth using our simple experimental system of isolated cerebellar granule neurons. CGN use glutamate as their neurotransmitter and thus do not synthesis acetylcholine from choline. In addition, phosphatidylethanolamine N-methyltransferase (PEMT), the enzyme which converts phosphatidylethanolamine to phosphatidylcholine, is expressed mainly in liver with very low expression in brain (Zhu and Zeisel, 2005). Hence, choline given to CGN can only be significantly metabolized to betaine or phosphocholine, suggesting that the protective properties of choline in CGN are mediated through membrane effects and/or changes in methionine synthesis or methylation. Several recent studies have investigated the changes in DNA methylation following ethanol and/or choline supplementation (Otero et al., 2012). Paradoxically, DNA methylation is reduced with choline supplementation following ethanol exposure making it unlikely that the only role choline plays is as a direct methyl donor.

Choline is also incorporated into membranes in the form of phosphatidylcholine and sphingomyelin. It is interesting to note in our results that in the absence of ethanol exposure, choline supplementation does not alter L1 signaling, L1 distribution in lipid rafts or neurite outgrowth with or without L1. Hence, choline alone does not appear to affect lipid raft function. Supplemental choline is taken up into cells by the choline transporter (Kuang et al., 2010), and rapidly synthesized to phosphatidylcholine and stored in the membrane (Gibellini and Smith, 2010). The rate limiting step is CTP:phosphocholine cytidylyltransferase (Gibellini and Smith, 2010). Cells become saturated with PtdCho, resulting in an efflux of choline from the cell or tissue (Klein et al., 1991). Thus, in the absence of ethanol, extra choline is stored in the membrane as PtdCho. We speculate that as the membrane concentrations of PtdCho increase, SM concentrations do not as sphingomyelin synthase is dependent on ceramide availability (Slotte, 2013). Thus as SM levels remains the same, the proportion of lipid ordered and lipid disordered membrane remains the same, and no effect on lipid raft function is observed. However, changes in lipid composition have been shown to modulate protein activity and trafficking (Dainese et al, 2014).

Exposure to ethanol has been shown to cause the rapid breakdown of SM to ceramide and pyrophosphate (Viktorov and Yurkiv, 2008). Ceramide is a pro-apoptotic signal (Saito and Saito, 2013). Loss of SM from lipid rafts could cause lipid raft dysfunction. In cells lacking sphingomyelin synthase which cannot synthesis SM, lipid raft dysfunction has been shown (Dong et al., 2012). Hence, a reduction in SM may cause lipid raft dysfunction. If there is stored PtdCho, ready conversion via sphingomyelin synthase may prevent a dysfunction of lipid rafts while SM is being broken down. In addition, choline given during or after the ethanol exposure may return the SM content back towards normal.

Previous work has shown that choline supplementation to fetal rats or rat pups given with or following ethanol exposure ameliorates but does not completely reverse, the impact of ethanol on neurobehavioral outcomes (Thomas and Tran, 2012; Thomas et al., 2010; Thomas et al., 2009; Ryan et al., 2008; Thomas et al., 2007; Thomas et al., 2004; Thomas et al., 2000; Monk et al., 2012; Monk et al., 2012). A longer time interval between ethanol exposure and choline supplementation reduces the neuroprotective effect of choline (Ryan et al., 2008). The mechanism of this neuroprotection by choline is not understood. Choline is an essential nutrient which is metabolized to 1) acetylcholine; 2) the membrane

phospholipids phosphatidylcholine and sphingomyelin, or 3) betaine which can be metabolized further to methionine, an important amino acid for protein synthesis, and S-adenosyl methionine, a methyl donor for a variety of molecules including DNA, RNA, and histones. Thus, mechanisms might include impacts on the cholinergic system, membrane function including signaling and/or methylation and protein synthesis. This study sought to examine the effects of choline supplementation on ethanol disruption of membrane function.

One unique aspect of our studies is that choline is given prior to ethanol treatment. We have focused on the acute effects of ethanol in our prior work (Yeaney et al., 2009; Bearer et al., 1999; Tang et al., 2006; Tang et al., 2011). The studies reported here use the same experimental model. As shown in this paper, choline pretreatment results in amelioration of ethanol's effects. It remains to be seen whether choline pretreatment improves cerebellar function *in vivo*. These results using supplemental choline, i.e. no effect on unexposed cells and amelioration of ethanol's effects on supplemented cells, raise the possibility of supplementing common foodstuffs as a public health strategy to reduce the impact of ethanol exposure on pregnant women regardless of their lifestyle choices. This strategy is currently being used successfully with folic acid supplementation of enriched pastas and bread to reduce neural tube defects (Obican et al., 2010), iodine addition to salt to prevent hypothyroidism (Pearce et al., 2013), and vitamin D to milk to prevent rickets (Shakur et al., 2014). Identifying pregnant women who drink and/or affected offspring is difficult (Astley, 2014; Gifford et al., 2010). It is intriguing to think that the relative deficiency of choline in the diet of pregnant women could be a risk factor for FASD. Several studies have shown that nutritional status is a confounding factor for FASD (Shankar et al., 2006; Abel, 2000; Abel, 2000; Abel and Hannigan, 1995; Shankar et al., 2007). The "enrichment" of common foodstuffs may have additional benefits from the amelioration of fetal alcohol exposure. Recent research on choline has led to a new definition of the Recommended Daily Allowance (RDA). For women, the RDA is 425 mg/day and for men 500 mg/day. Pregnant and lactating women have higher RDAs of 450 mg/d and 550 mg/d respectively. An analysis of NHANES 2003–2004 revealed that less than 10% of the adult population attained these RDA's (Jensen et al., 2007). And in the 2007–2008 NHANES, the mean average daily intake for adults was 302 mg/d (Chester et al., 2011). Several genetic polymorphisms make the ADI larger in some individuals (da Costa et al., 2014) who would thus be benefitted by additional choline in their diet.

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References

- Abel EL. The role of dietary fat in alcohol's prenatal effects. *Alcohol*. 2000; 20:83–86. [PubMed: 10680721]
- Abel EL, Hannigan JH. Maternal risk factors in fetal alcohol syndrome: Provocative and permissive influences. *Neurotoxicology & Teratology*. 1995; 17:445–462. [PubMed: 7565491]
- Astley SJ. Invited commentary on Australian fetal alcohol spectrum disorder diagnostic guidelines. *BMC Pediatr*. 2014; 14:85–2431-14-85. [PubMed: 24685275]

- 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]
- Blanco AM, Perez-Arago A, Fernandez-Lizarbe S, Guerri C. Ethanol mimics ligand-mediated activation and endocytosis of IL-1RI/TLR4 receptors via lipid rafts caveolae in astroglial cells. *J Neurochem*. 2008; 106:625–639. [PubMed: 18419766]
- Charness ME, Safran RM, Perides G. Ethanol inhibits neural cell-cell adhesion. *Journal of Biological Chemistry*. 1994; 269:9304–9309. [PubMed: 8132668]
- Chester, DN.; Goldman, JD.; Ahuja, JK.; Moshfegh, AJ. FoodSurveys Research Group Dietary Data Brief No. 9. 2011. Dietary intakes of choline: What we eat in America, NHANES 2007–2008.
- da Costa KA, Corbin KD, Niculescu MD, Galanko JA, Zeisel SH. Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. *FASEB J*. 2014; 28:2970–2978. [PubMed: 24671709]
- Dainese E, De Fabritiis G, Sabatucci A, Oddi S, Angelucci CB, Di Pancrazio C, Giorgino T, Stanley N, Del Carlo M, Cravatt BF, Maccarrone M. Membrane lipids are key modulators of the endocannabinoid-hydrolase FAAH. *Biochem J*. 2014; 457:463–472. [PubMed: 24215562]
- Dong L, Watanabe K, Itoh M, Huan CR, Tong XP, Nakamura T, Miki M, Iwao H, Nakajima A, Sakai T, Kawanami T, Sawaki T, Masaki Y, Fukushima T, Fujita Y, Tanaka M, Yano M, Okazaki T, Umehara H. CD4+ T-cell dysfunctions through the impaired lipid rafts ameliorate concanavalin A-induced hepatitis in sphingomyelin synthase 1-knockout mice. *Int Immunol*. 2012; 24:327–337. [PubMed: 22345277]
- Fransen E, D’Hooge R, Van Camp G, Verhoye M, Sijbers J, Reyniers E, Soriano P, Kamiguchi H, Willemsen R, Koekkoek SKE, De Zeeuw CI, De Deyn PP, Van der Linden A, Lemmon V, Kooy RF, Willems PJ. L1 knockout mice show dilated ventricles, vermis hypoplasia and impaired exploration patterns. *Human Molecular Genetics*. 1998; 7:999–1009. [PubMed: 9580664]
- Fransen E, Lemmon V, Van Camp L, Vits P, Coucke P, Willems PJ. CRASH syndrome: a clinical spectrum of corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraparesis and hydrocephalus due to mutations in one single gene, L1. *European Journal of Human Genetics*. 1995; 3:273–284. [PubMed: 8556302]
- Fransen E, Vits L, Van Camp G, Willams PJ. The clinical spectrum of mutations in L1, a neuronal cell adhesion molecule. *American Journal of Medical Genetics*. 1996; 64:73–77. [PubMed: 8826452]
- Gibellini F, Smith TK. The Kennedy pathway--De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life*. 2010; 62:414–428. [PubMed: 20503434]
- Gifford AE, Farkas KJ, Jackson LW, Molteno CD, Jacobson JL, Jacobson SW, Bearer CF. Assessment of benefits of a universal screen for maternal alcohol use during pregnancy. *Birth Defects Res A Clin Mol Teratol*. 2010; 88:838–846. [PubMed: 20890939]
- Havens JR, Simmons LA, Shannon LM, Hansen WF. Factors associated with substance use during pregnancy: results from a national sample. *Drug Alcohol Depend*. 2009; 99:89–95. [PubMed: 18778900]
- 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).
- Klein J, Koppen A, Loffelholz K. Uptake and storage of choline by rat brain: influence of dietary choline supplementation. *J Neurochem*. 1991; 57:370–375. [PubMed: 2072091]
- Koc H, Mar M-H, Ranasinghe A, Swenberg JA, Zeisel SH. Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. *Anal Chem*. 2002; 74:4734–4740. [PubMed: 12349977]
- Kuang Y, Salem N, Corn DJ, Erokwu B, Tian H, Wang F, Lee Z. Transport and metabolism of radiolabeled choline in hepatocellular carcinoma. *Mol Pharm*. 2010; 7:2077–2092. [PubMed: 20698576]
- 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]

- Maness PF, Schachner M. Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. *Nat Neurosci.* 2007; 10:19–26. [PubMed: 17189949]
- May PA, Blankenship J, Marais AS, Gossage JP, Kalberg WO, Barnard R, De Vries M, Robinson LK, Adnams CM, Buckley D, Manning M, Jones KL, Parry C, Hoyme HE, Seedat S. Approaching the prevalence of the full spectrum of fetal alcohol spectrum disorders in a South African population-based study. *Alcohol Clin Exp Res.* 2013; 37:818–830. [PubMed: 23241076]
- 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]
- Nakai Y, Kamiguchi H. Migration of nerve growth cones requires detergent-resistant membranes in a spatially defined and substrate-dependent manner. *Journal of Cell Biology.* 2002; 159:1097–1108. [PubMed: 12499360]
- Obican SG, Finnell RH, Mills JL, Shaw GM, Scialli AR. Folic acid in early pregnancy: a public health success story. *FASEB J.* 2010; 24:4167–4174. [PubMed: 20631328]
- Otero NK, Thomas JD, Saski CA, Xia X, Kelly SJ. Choline supplementation and DNA methylation in the hippocampus and prefrontal cortex of rats exposed to alcohol during development. *Alcohol Clin Exp Res.* 2012; 36:1701–1709. [PubMed: 22509990]
- Pearce EN, Andersson M, Zimmermann MB. Global iodine nutrition: Where do we stand in 2013? *Thyroid.* 2013; 23:523–528. [PubMed: 23472655]
- Pike LJ. The challenge of lipid rafts. *J Lipid Res.* 2009; 50(Suppl):S323–8. [PubMed: 18955730]
- Ramanathan R, Wilkemeyer M, Mittal B, Perides G, Charness M. Alcohol inhibits cell-cell adhesion mediated by human L1. *Journal of Cell Biology.* 1996; 133:381–390. [PubMed: 8609170]
- 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]
- Saito M, Saito M. Involvement of sphingolipids in ethanol neurotoxicity in the developing brain. *Brain Sci.* 2013; 3:670–703. [PubMed: 24961420]
- Sampson PD, Streissguth AP, Bookstein FL, Little RE, Clarren SK, Dehaene P, Hanson JW, Graham MJM. Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology.* 1997; 56:317–326. [PubMed: 9451756]
- Schaefer AW, Kamei Y, Kamiguchi H, Wong EV, Rapoport I, Kirchhausen T, Beach CM, Landreth G, Lemmon SK, Lemmon V. L1 endocytosis is controlled by a phosphorylation-dephosphorylation cycle stimulated by outside-in signaling by L1. *Journal of Cell Biology.* 2002; 157:1223–1232. [PubMed: 12082080]
- Schaefer AW, Kamiguchi H, Wong EV, Beach CM, Landreth G, Lemmon V. Activation of the MAPK signal cascade by the neural cell adhesion molecule L1 requires L1 internalization. *Journal of Biological Chemistry.* 1999; 274:37965–37967. [PubMed: 10608864]
- Schmid RS, Pruitt WM, Maness PF. A MAP kinase-signaling pathway mediates neurite outgrowth on L1 and requires Src-dependent endocytosis. *Journal of Neuroscience.* 2000; 20:4177–4188. [PubMed: 10818153]
- Sebastian BM, Nagy LE. Decreased insulin-dependent glucose transport by chronic ethanol feeding is associated with dysregulation of the Cbl/TC10 pathway in rat adipocytes. *Am J Physiol Endocrinol Metab.* 2005; 289:E1077–84. [PubMed: 16105861]
- Shakur YA, Lou W, L'Abbe MR. Examining the effects of increased vitamin D fortification on dietary inadequacy in Canada. *Can J Public Health.* 2014; 105:e127–32. [PubMed: 24886848]
- Shankar K, Hidestrand M, Liu X, Xiao R, Skinner CM, Simmen FA, Badger TM, Ronis MJ. Physiologic and genomic analyses of nutrition-ethanol interactions during gestation: Implications for fetal ethanol toxicity. *Exp Biol Med (Maywood).* 2006; 231:1379–1397. [PubMed: 16946407]
- Shankar K, Ronis MJ, Badger TM. Effects of pregnancy and nutritional status on alcohol metabolism. *Alcohol Res Health.* 2007; 30:55–59. [PubMed: 17718402]
- Slotte JP. Biological functions of sphingomyelins. *Prog Lipid Res.* 2013; 52:424–437. [PubMed: 23684760]

- Stratton, K.; Howe, C.; Battaglia, F., editors. Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment. National Academy Press; Washington, D.C: 1996. Summary.
- Szabo G, Dolganiuc A, Dai Q, Pruett SB. TLR4, ethanol, and lipid rafts: a new mechanism of ethanol action with implications for other receptor-mediated effects. *J Immunol.* 2007; 178:1243–1249. [PubMed: 17237368]
- 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]
- Tang N, He M, O’Riordan MA, Farkas C, Buck K, Lemmon V, Bearer CF. Ethanol inhibits L1 cell adhesion molecule activation of mitogen-activated protein kinases. *J Neurochem.* 2006; 96:1480–1490. [PubMed: 16478533]
- 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]
- 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]
- 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]
- 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]
- 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]
- 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]
- Tsui-Pierchala BA, Encinas M, Milbrandt J, Johnson EM Jr. Lipid rafts in neuronal signaling and function. *Trends Neurosci.* 2002; 25:412–417. [PubMed: 12127758]
- Tuvia S, Garver T, VB. The phosphorylation state of the FIGQY tyrosine of neurofascin determines ankyrin-binding activity and patterns of cell segregation. *Proceedings of the National Academy of Science U S A.* 1997; 94:12957–12962.
- Viktorov AV, Yurkiv VA. Effects of ethanol and lipopolysaccharide on the sphingomyelin cycle in rat hepatocytes. *Bull Exp Biol Med.* 2008; 146:753–755. [PubMed: 19513375]
- Watanabe H, Ymazaki M, Miyazaki H, Arikawa C, Itoh K, Sasaki T, Maehama T, Frohman MA, Kanaho Y. Phospholipase D2 functions as a downstream signaling molecule of MAP kinase pathway in L1-stimulated neurite outgrowth of cerebellar granule neurons. *Journal of Neurochemistry.* 2004; 89:142–151. [PubMed: 15030398]
- Yeaney NK, He M, Tang N, Malouf AT, O’Riordan MA, Lemmon V, Bearer CF. Ethanol inhibits L1 cell adhesion molecule tyrosine phosphorylation and dephosphorylation and activation of pp60(src). *J Neurochem.* 2009; 110:779–790. [PubMed: 19457108]
- Zhu X, Zeisel SH. Gene expression profiling in phosphatidylethanolamine N-methyltransferase knock out mice. *Brain Res Mol Brain Res.* 2005; 134:239–255. [PubMed: 15836921]

Figure 1.

Choline reduces the effect of ethanol in concentrations greater than 20 μ M. CGN from a single cell preparation were plated in serum free defined media on poly L-lysine. The following additives were added after 2 hours: L1-Fc (L1), ethanol (EtOH) 25 mM, or choline chloride in the concentrations indicated. Cells were fixed and immunostained at 24 h, and neurite length measured. Shown is mean $\pm$ SEM of all neurites on a single coverslip. * $p < 0.0001$ vs PLL alone, ** $p < 0.0001$ vs L1 alone, 2 tailed t-test.

Figure 2.

Acute ethanol did not alter choline content. CGN were cultured for 24h in complete media with or without 40 μM choline chloride (Cho). 1 h prior to harvest, 25 mM ethanol (EtOH) was added. Cells were washed 3x in HBSS, lysed in RIPA buffer, and intracellular choline content determined. Shown is mean $\pm$ SD, * p <0.02 vs Ctrl, ** p <0.05 vs Ctrl, 2 tailed t-test.

Figure 3.

Choline significantly reduces the inhibition of ethanol on L1 stimulated tyrosine phosphorylation. CGN were culture on PLL for 24 h in complete media with or without choline, then serum starved for 1 h, followed by the addition of 25 mM ethanol. Cross-linked monoclonal antibody to the extracellular domain of L1 (ASCS4) was added, and cells were harvested 10 min later. Lysates were immunoprecipitated using a polyclonal antibody to the cytoplasmic domain of L1 (L1CD), followed by immunoblot with a monoclonal antibody to phosphotyrosine (PY100). Blots were stripped and reblotted with L1CD as a loading control. A) Representative blot. B) Relative densitometric units shown as mean $\pm$ SD. *PLL vs ASCS4; *ASCS4 vs ASCS4+Ethanol; *ASCS4+Ethanol+Choline vs ASCS4+Ethanol; *ASCS4+Ethanol+Choline vs ASCS4, $p < 0.03$; 2 tailed t-test.

Figure 4.

Choline significantly reduces the inhibition by ethanol of L1 mediated ERK1/2 activation. CGN were treated as described in Figure 3. Cell lysates were immunoblotted with antibody to phosphoERK1/2, then stripped and reblotted for total ERK1/2 as a loading control. A) Representative blot. B) Relative densitometric units shown as mean $\pm$ SD. *PLL vs ASCS4, $p < 0.01$; **ASCS4 vs ASCS4+Ethanol, $p < 0.02$, ***ASCS4+Ethanol+Choline vs ASCS4 +Ethanol and vs ASCS4 alone, $p < 0.03$; 2 tailed t-test.

Figure 5.

Choline significantly reduces the inhibition by ethanol of L1 activation of pp60^{src}. CGN were treated as described in Figure 3. Lysates were immunoprecipitated with a mouse monoclonal antibody to pp60^{src}, then immunoblotted for the active form of pp60^{src} where it is phosphorylated on tyrosine 416 (P416). Blots were stripped and reblotted for total pp60^{src}.

A) Representative immunoblot. B) Relative densitometric units shown as mean $\pm$ SD.

*PLL vs ASCS4, $p < 0.02$; **ASCS4 vs ASCS4+Ethanol, $p < 0.05$, ***ASCS4+Ethanol +Choline vs ASCS4 +Ethanol and vs ASCS4 alone, $p < 0.03$; 2 tailed t-test.

Figure 6.

Choline significantly reduces the inhibition by ethanol of L1 dephosphorylation on Y1176. CGN were treated as described in Figure 3. Cell lysates were immunoblotted with antibody to dephosphorylated Y1176-L1 (74-5H7), then stripped and reblotted for total ERK1/2 as a loading control. A) Representative blot. B) Relative densitometric units shown as mean $\pm$ SD. *PLL vs ASCS4, $p < 0.01$; **ASCS4 vs ASCS4+Ethanol, $p < 0.002$, ***ASCS4+Ethanol +choline vs ASCS4 +Ethanol and vs ASCS4 alone, $p < 0.01$; 2 tailed t-test.

Figure 7.

Choline pretreatment significantly reduces the redistribution of L1 into lipid rafts in the presence of ethanol. CGN were plated on PLL for 24 h in complete media and supplemented with 40 μ M choline (Cho). 25 mM ethanol (EtOH) was added for 1 h, then cell lysed and lipid rafts isolated. Immunoblots using polyclonal antibody to the cytoplasmic domain of L1 (L1CD) were prepared with equivalent volumes of lipid raft (LR) and non-lipid raft pools (N). Blots were stripped and reblotted for transferrin receptor (TfR) as a marker for non-lipid rafts, then stripped and reblotted with cholera toxin B (CTxB) which recognizes GM1 ganglioside as a marker of lipid rafts to show that the separation of lipid rafts and non-lipid raft proteins was complete. A) Representative immunoblot. B) The percent of L1 in the lipid

raft pool was calculated by measuring the pixel density in both the LR and N lanes, then $LR/(LR+N) \times 100$ was determined. Ctrl – no choline, no ethanol; EtOH-CGN treated with 25 mM ethanol alone; Cho + EtOH – CGN treated with both choline and ethanol; Cho-cells treated with choline alone. *EtOH vs Ctrl, $p<0.005$; **EtOH vs Cho+EtOH, $p<0.02$; ***Cho+EtOH vs Ctrl, $p<0.05$; 2 tailed t-test.

Figure 8.

Choline significantly increases neurite length of CGN grown on L1 in the presence of ethanol. CGN were plated on poly L-lysine (PLL) and treated as described in Figure 1. Additives were L1-Fc (L1), 25 mM ethanol (EtOH), 40 μ M choline (Cho). * vs PLL, $p < 0.02$, ** vs L1, $p < 0.02$, *** vs L1 + EtOH, vs L1 alone, $p < 0.05$; 2 tailed t-test.