Published in final edited form as:

Neuroscience. 2011 April 28; 180: 30–40. doi:10.1016/j.neuroscience.2011.02.018.

L1CAM promotes resistance to alcohol-induced silencing of growth cone responses to guidance cues

Bryan Sepulveda¹, Ioana Carcea¹, Becky Zhao¹, Stephen R.J. Salton^{1,2}, and Deanna L. Benson^{1,3}

¹Fishberg Department of Neuroscience, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029

²Brookdale Department of Geriatrics and Palliative Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029

Abstract

Alcohol exposure *in utero* is a common cause of mental retardation, but the targets and mechanisms of action are poorly understood. Several lines of data point toward alterations in cortical connectivity, suggesting that axon guidance may be vulnerable to alcohol exposure. To test this, we asked whether ethanol directly affects cortical axonal growth cone responses to guidance cues. We find that even low concentrations of ethanol (12.5 mM; 57.2mg/dL) commonly observed in social drinking prevent growth cone responses to three mechanistically independent guidance cues, Semaphorin3A, Lysophosphatidic Acid, and Netrin-1. However, this effect is highly dependent on substrate; axonal growth cones extending on an L1CAM substrate retain responsiveness to cues following exposure to ethanol, while those growing on poly-L-lysine or N-cadherin do not. The effects of ethanol on axon extension are, by contrast, quite modest. Quantitative assessments of the effects of ethanol on the surface distribution of L1CAM in growth cones suggest that L1CAM homophilic interactions may be particularly relevant for retaining growth cone responsiveness following ethanol exposure. Together, our findings indicate that ethanol can directly and generally alter growth cone responses to guidance cues, that a substrate of L1CAM effectively antagonizes this effect, and that cortical axonal growth cone vulnerability to ethanol may be predicted in part based on the environment through which they are extending.

Keywords

ethanol; axon guidance; L1CAM; N-cadherin; Sema3A; Netrin

© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

³**Corresponding Author** Fishberg Department of Neuroscience, Box 1065 The Mount Sinai School of Medicine One Gustave L. Levy Place, Box 1065, New York, NY 10029 Tel.: (212) 659-5906 Fax: (212) 996-9785 Deanna.Benson@mssm.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

*Research Highlights

- Ethanol prevents growth cone responses to guidance cues, Sema3A, Netrin. and LPA.
- Axons extending on an L1CAM substrate selectively retain responsiveness to cues.
- L1CAM binding preserves L1CAM localization in growth cones exposed to ethanol.

Introduction

Fetal alcohol spectrum disorders (FASD), which include fetal alcohol syndrome (FAS), occur as a result of alcohol exposure *in utero* and are among the most common causes of mental retardation (Jones and Smith, 1973). The cerebral cortex is particularly vulnerable, but the mechanisms by which alcohol mediates its devastating effects remain poorly understood. In animal models of FASD, early exposure to ethanol can alter normal patterns of cortical neuronal migration (Cuzon et al, 2008; Miller, 1986; Siegenthaler and Miller, 2004). Axon growth toward a target shares several mechanisms with neuron migration and as such, would be predicted to be sensitive to ethanol exposure, but this has not been tested experimentally.

Studies in humans suggest that prenatal exposure to ethanol disrupts the development of normal axon trajectories in neocortex. Diffusion tensor imaging (DTI), a noninvasive MRI-based technique used to assess the integrity of axon tracts shows that exposure to alcohol correlates with significant alterations in the corpus callosum (Li et al, 2009; Ma et al, 2005; Wozniak et al, 2006, 2009), as well as with abnormalities in the cingulum and fiber tracts joining temporal and frontal cortices (Fryer et al, 2009; Lebel et al, 2008; Sowell et al, 2008) that cannot be fully accounted for by reduced myelination (Sowell et al, 2008). Additionally, work in rat models of FASD has shown that *in utero* ethanol exposure alters the development and maturation of connectivity in sensory motor cortex (Chappell et al, 2007; Margret et al, 2005, 2006a, 2006b; Miller and al-Rabiai, 1994). In rat hippocampus, prenatal exposure to ethanol alters mossy fiber guidance (West et al, 1981), and there is some indication that in cultured hippocampal neurons, growth cone attraction toward a gradient of BDNF may be reversed by exposure to ethanol (Lindsley et al, 2006). Together these data suggest that ethanol exposure may alter the course of developing cortical axons, but whether or how ethanol affects growth cone responses to guidance cues has not been addressed.

During cortical development, axonal growth cones navigate to their targets by responding to strategically placed cues that either attract or repel and thereby, guide them. To extend in the appropriate direction, actin-linked cell adhesion molecules (CAMs) expressed on growth cone surfaces dynamically interact with particular substrates to generate traction and move forward. CAMs can also act as receptors or co-receptors for cues or they can modify the actions of guidance cue receptors (e.g. Castellani et al, 2000; Paratcha et al, 2003), making them an inextricable part of growth and guidance. Significantly, several CAMs have been implicated in FASD pathology (Miñana et al, 2002; Ramanathan et al, 1996; Siegenthaler and Miller, 2004). With this in mind, we asked whether exposure to ethanol altered cortical axonal growth cone responses to several well-characterized cues and whether responses could be modified by different substrates.

Experimental Procedures

Cell Culture Preparation

Embryonic day 18 (E18) rat neocortical neurons were dissociated and plated at a density of 2×10^5 cells per plate (1.4×10^4 cells/cm²) on cover slips coated with poly-L-lysine (PLL), human L1CAM-Fc or mouse N-cadherin-Fc (Ncad) using protocols previously described (Dickson et al, 2002). Neurons were allowed to adhere for about three hours in media containing 10% fetal bovine serum and then they were transferred to Neurobasal Media containing B27 (Invitrogen) or NS21 (Chen et al., 2008) in a 5% CO₂, 37°C incubator for three to four days. All results reported represent data from two or more different cultures.

Ethanol exposure

Neurons were first exposed to ethanol by transferring the cover slips to sterile, 12-well polyethylene plates containing ethanol and/or Neurobasal. The ethanol concentrations used were: 0 mM, equivalent to 0% Blood Alcohol Content (BAC), 12.5 mM (0.725 μ l/ml 100% EtOH; 0.0572% BAC), 25 mM (1.450 μ l/ml 100% EtOH; 0.1144% BAC), and 50 mM (2.900 μ l/ml 100% EtOH; 0.2288% BAC) (Brick, 2006). Neurons that were exposed to ethanol were incubated in their respective ethanol solutions for one hour in a 5% CO₂, 37°C incubator containing a water bath with 50 mM ethanol in order to reduce ethanol loss due to evaporation (Bearer et al, 1999). One hour was chosen because it was the time point at which axonal motility was maximally inhibited in live imaging experiments. Neurons that were not exposed to ethanol (control) were placed in an identical incubator with an ethanol-free water bath. Cover slips were then briefly (< 1 min) washed in Neurobasal to remove excess ethanol.

Collapse assays

Growth cone collapse in response to Sema3A or LPA was carried out based on parameters described previously (Mintz et al, 2008). Neurons were transferred to 12-well plates containing 100 ng/ml recombinant Sema3A-Fc (R&D Systems), 1 μ M LPA (Sigma), or Neurobasal (control) for 20-30 mins. The neurons were then fixed in 2% paraformaldehyde / 2% sucrose pre-warmed to 37°C, permeabilized with 0.25% Triton X-100 and labeled with rhodamine-conjugated phalloidin (Molecular Probes; 1:100).

For collapse in response to Netrin, neurons plated on laminin (Dent et al, 2004; Hopker et al, 1999) were incubated in ethanol (0, 12.5, 25 or 50 mM) with or without Netrin (R&D Systems; 250 ng/ml) in a sealed chamber together for 7 h, a time course over which the collapse response was sustained and additional parameters could be analyzed (Dent et al, 2004; Mintz et al, unpublished data). Neurons were then fixed and stained as described above.

As a control to confirm that ethanol mediates its effects on growth cones rather than the substrate upon which the neurons were plated, the collapse assays were conducted as described above following an additional washout of 1 h in control media in the incubator. Previous work has shown that growth cone motility and normal axonal morphology is typically restored after washout of 1 h of Sema3A (Kapfhammer et al, 2007). This control was done for Sema3A and LPA on PLL, L1CAM and Ncad and for Netrin on laminin at 0 and 50mM EtOH. At least 100 growth cones were counted per coverslip in two different cultures.

Axons were identified as the longest neurite, exceeding all other processes in length by at least 10 μ m (Goslin and Banker, 1989) and their growth cones were imaged on a Zeiss Axiophot fluorescent microscope using a 63X objective. Growth cones were counted and classified as either extended or collapsed; those containing less than three filopodia extending away from the general direction of the axon's extension were classified as collapsed (Mintz et al, 2008). At least 100 growth cones were counted and classified for each cover slip, and the proportion of growth cone collapse was calculated. Statistical comparisons between groups were made using Prism (GraphPad Software).

For collapse assays either 1 or 2-way ANOVAs were used to analyze the effect of the substrates and ethanol. Tukey's (1-way) or Bonferroni (2-way) post tests were used to compare effect of substrates at the same ethanol concentration. The tests used along with experimental number are indicated in the text and figure legends.

Time lapse imaging

Neurons were grown in PLL-coated 12-well plates. They were exposed to 0 (6 wells) or 25mM ethanol (6 wells) and imaged using the Live Cell Olympus IX-70 microscope in a total volume that filled the well, which was then sealed with vacuum grease and a sterile coverslip to prevent the evaporation of ethanol, which was present throughout imaging. Within 1 h or 3 h (see results) of the onset of ethanol exposure, images of three separate fields in each well were taken with a 20X objective. Images were then taken every 15 mins for a total of 1260 mins (21 h). Axon growth was analyzed quantitatively using Image J (NIH) and MetaMorph (Molecular Devices; Downingtown, PA) software. All axons that remained within the field of view for the entire recording period and did not fasciculate were analyzed. In the results, we report axonal outgrowth velocity (the absolute length divided by the elapsed time), the cumulative overall outgrowth (the absolute length regardless of direction) and the vector distance, which measures directed outgrowth and reveals the axonal length at the end of the study period. For each parameter, the two groups were compared in paired, two tailed, t-tests. Numbers are provided in the figure legends.

Immunocytochemistry

Neurons (3 days old) were exposed to the indicated ethanol concentrations and then fixed in 2% paraformaldehyde/ 2% sucrose as described above. To label surface L1CAM, neurons were exposed to a mouse anti-L1CAM (ASCS4; Developmental Studies Hybridoma Bank, Paul Patterson; 1:50) that recognizes an epitope in the extracellular domain, but does not recognize the human L1CAM-Fc substrate on the coverslips (Mintz et al, 2008; Tang et al, 2006) for 20 mins after which they were fixed under nonpermeabilizing conditions and then incubated with an anti-mouse secondary antibody. GM1-enriched membrane microdomains were labeled in living neurons using fluorescein isothiocyanate (FITC)-conjugated cholera toxin subunit B (CtxB). Flotillin-2 was labeled using mouse anti-Flotillin-2 (BD Biosciences, San Jose, CA, 1:100) overnight at 4° C in neurons that had been fixed and then permeabilized with 0.25% Triton X-100. Primary antibody labeling was visualized with the appropriate fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch; 1:200). Confocal images were collected on an LSM 510 Zeiss microscope using a 100X objective using the same acquisition parameters (brightness, contrast, laser settings, filters) across images in order to accurately measure fluorescence intensities.

Fluorescence intensity distribution assays

Using MetaMorph, linescan intensity measurements were taken to assay fluorophore intensity along the length of growth cones. This was done for neurons grown on PLL, L1CAM or Ncad in the presence or absence of 50 mM ethanol for 1 h. Three linescan measurements were taken from the base, defined as the point from which the axon expands and does not become thinner (Bovolenta et al, 1987), to the filopodial tips. Lengths were normalized in order to compare the distribution of intensity measurements between growth cones at the same relative positions. For all three immunolabels (L1CAM, CtxB, Flotillin-2), a Kruskal-Wallis test was used to compare cumulative histogram frequencies across all three substrates, in addition to a Dunn's post test.

Results

Ethanol inhibits growth cone responses to Sema3A

We asked whether ethanol exposure alters growth cone responses to guidance cues. To test this, we first measured growth cone collapse in response to Sema3A, a repulsive guidance cue expressed in the developing cortex. Collapse is thought to be closely related to repulsive guidance, and can be readily quantified in large populations of growth cones (Kapfhammer

et al, 2007; Fig. 1A, B). Ethanol alone did not alter baseline levels of growth cone collapse, but it prevented collapse in response to Sema3A even at 12.5 mM (0.0572% BAC) and to a greater extent at higher concentrations (Fig. 1C) (2-way ANOVA and Bonferroni post test, see figure legend for details). Similar results were obtained when Sema3A was added together with ethanol (data not shown), indicating that the effects of ethanol are fast and lasting after washout. Additionally, after 1 h washout of Sema3A, the percentage of collapsed growth cones on PLL returned to baseline values, indicating that neither ethanol nor the cue altered the substrate (2-way ANOVA, $p > 0.10$ for interaction, exposure to cue or ethanol).

L1CAM substrates preserve growth cone responses to Sema3A

Several studies have shown that the substrate on which neurons grow can affect their responses to ethanol (Bearer et al, 1999, 2001; Hoffman et al, 2008). To determine the effect of substrate on ethanol-mediated inhibition of growth cone responses to guidance cues, we plated neurons on PLL (an artificial substrate), Ncad, or L1CAM (abundant endogenous substrates) and measured growth cone collapse in response to Sema3A. In the absence of ethanol, growth cones on all three substrates responded similarly to Sema3A (Fig. 1D). After exposure to 12.5 and 50 mM ethanol, the percentage of Sema3A-collapsed growth cones decreased to control levels (i.e. on PLL without Sema3A). Ncad substrates did not alter this effect of ethanol. In contrast, neurons growing on L1CAM displayed a normal collapse response to Sema3A at all concentrations of ethanol tested; collapse on L1CAM was insignificantly decreased relative to PLL (Fig. 1D) (2-way ANOVA and Bonferroni post test, see figure legend for details). There was no effect of ethanol on recovery from collapse following washout of Sema3A on either Ncad or L1CAM substrates (2-way ANOVAs, $p > 0.30$ for interactions, exposures to cue or ethanol).

Ethanol generally prevents growth cone collapse in response to guidance cues

Ethanol interferes with L1CAM binding in certain cell lines (Charness et al, 1994; Ramanathan et al, 1996; Wilkemeyer and Charness, 1998), and since L1CAM is a necessary co-receptor for Sema3A (Castellani et al, 2000), responses to Sema3A may be selectively targeted by ethanol. If this were the case, we would anticipate that responses to other guidance cues would be unaffected. To test this, we measured the impact of ethanol on growth cone collapse in response to Netrin, an unrelated guidance cue that can stimulate growth cone collapse in neurons grown on laminin (Hopker et al, 1999). Exposure to 250 ng/mL Netrin resulted in a 1.5 fold increase in cortical growth cone collapse in neurons grown on laminin compared to those on PLL (Fig. 2A) (one-way ANOVA and Tukey's post test, see figure legend for details). Ethanol significantly inhibited Netrin-mediated growth cone collapse on laminin at all concentrations examined. Following washout of Netrin for 1h, growth cones recovered similarly and irrespective of prior exposure to ethanol (ANOVA, $p = 0.80$). These data indicate that ethanol generally inhibits growth cone responsiveness to guidance cues.

L1CAM generally preserves growth cone responsiveness

We also tested growth cone collapse in neurons grown on PLL, Ncad or L1CAM in response to LPA, a mechanistically distinct cue that also induces cortical growth cone collapse similar to Sema3A (Fukushima et al, 2002; Jalink et al, 1994). Ethanol reduced LPA-mediated growth cone collapse in neurons grown on PLL and on Ncad but had no significant impact on neurons grown on L1CAM (Fig. 2B) (2-way ANOVA and Bonferroni post test, see figure legend for detail). Additionally, growth cones recover following washout of LPA (2-way ANOVA, $p > 0.10$ for interaction, exposure to cue or ethanol). These data support that L1CAM generally preserves growth cone responsiveness to guidance cues in the presence of ethanol.

Ethanol has modest impact on axon growth

In previous work, we have shown that 24-48 h of continuous exposure to ethanol has little impact on cortical axon extension in neurons grown on either PLL or L1CAM (Hoffman et al, 2008). In the present study, we assayed the effects of ethanol on axon guidance, a process that is mechanistically distinct from extension (Bentley and Toroian-Raymond, 1986; Chien et al, 1993) following acute, 1 h, exposure. In order to bridge the two sets of data and distinct exposure conditions, we asked whether ethanol differentially regulates cortical axon outgrowth over the course of development, similar to what has been described in hippocampal neurons (Lindsley et al, 2003). Time-lapse imaging was used to document axon growth on PLL in control cortical neurons or those cultured in the presence of 25 mM ethanol for 21 h, a time period covering both acute and long term observations. Differences between groups were compared using paired, two-tailed t-tests. For a period ranging from 3 h - 21 h, ethanol significantly reduced the mean velocity of axon extension compared to control, but this translates into only a very modest decrease in axon length, particularly when compared to the impact of ethanol on other cell types (Bearer et al, 1999) (Fig. 3A, B). Additionally, the average rate of growth was constant over time in both conditions. However, ethanol did alter the overall growth trajectory. This was evaluated by measuring growth cone displacement from their points of origin at the start of the experiment. The data show that axons exposed to ethanol ranged significantly closer to their start site (Fig. 3C).

In the above experiments, imaging was initiated 3 h post-ethanol addition, and it remained possible that we missed an acute period of greater disparity between conditions. To address this, neurons were imaged beginning at 1 h following the addition of ethanol and continuing to 3 h. Under these conditions, the results were quite similar to longer exposure times: velocity and axon extension were very modestly inhibited by ethanol (Fig. 3D, E), while displacement was substantially and significantly reduced by ethanol in the first three hours of imaging (Fig. 3F). Thus, ethanol has a sustained negative effect on growth that can be detected using time lapse imaging (a more sensitive method than the post hoc analyses we employed previously, Hoffman et al, 2008), but this effect is insufficient to account for the dramatic inhibition that ethanol shows on growth cone responses to guidance cues.

L1CAM substrate preserves surface L1CAM

Axonal growth cones extending on L1CAM can better maintain responsiveness to guidance cues in the presence of ethanol, but the mechanism is unclear. Since L1CAM is very abundant in axons and can bind itself, homophilic interactions could potentially organize or stabilize growth cone membranes. If this were the case, one would predict that following exposure to ethanol L1CAM distribution in growth cones growing on L1CAM would be more stable than on other substrates. To test this, we determined the baseline distribution of L1CAM in growth cones on different substrates and then assessed the impact of ethanol on L1CAM distribution.

Neurons were cultured on PLL, L1CAM or Ncad substrates, fixed under non-permeabilizing conditions and labeled for L1CAM. On all three substrates, and in both central and peripheral growth cone domains, faint and diffuse L1CAM labeling was accompanied by more intensely labeled clusters. Since growth cone size and shape can vary on different substrates, localization and intensity were compared using line scans drawn from base to growth cone tip (see Methods). On all three substrates, L1CAM showed a proximal to distal gradient that was shallowest on Ncad (Fig. 4). Exposure to ethanol did not alter this general localization pattern on any of the substrates (Fig. 4).

In contrast, there were notable changes in labeling intensity that were substrate- and ethanol-dependent. This measure reflects surface levels and/or the extent of surface clustering.

Intensities were plotted as cumulative distributions and compared using a Kruskal-Wallis test and a Dunn's post test. The cumulative distribution of L1CAM intensity (Fig. 5A) was similar on PLL (Fig. 5D) and Ncad (Fig. 5G), with modes just below 100 intensity units, but those on L1CAM were shifted towards higher intensities, with the mode at 140 intensity units. On the L1CAM substrate, the distribution of L1CAM labeling is largely preserved after exposure to 50 mM ethanol: there is an insignificant decrease in intensity that is limited to regions showing highest labeling intensity (Fig. 5A). By contrast, exposure to ethanol produced significant increases in the intensities of L1CAM labeling in growth cones on PLL (Fig. 5D-F) and on Ncad (Fig. 5G-I) compared to their respective controls.

Appropriate responses to Sema3A, Netrin-1 (Guirland et al, 2004) and LPA (Peres et al, 2003) require recruitment of their respective receptors into GM1-enriched membrane microdomains or lipid rafts. Therefore, we asked whether Cholera toxin B (CtxB), which binds GM1, would also show a substrate dependent response to ethanol exposure. Baseline intensity distributions of CtxB on L1CAM (Fig. 5J) and Ncad (Fig. 5L) were similar, but in response to ethanol, the intensity distributions in all growth cones shifted to higher levels. On PLL, the baseline intensity distribution of CtxB started at higher values than those for L1CAM and Ncad without ethanol, and was shifted toward lower values in the presence of ethanol (Fig. 5K). We also examined Flotillin-2, which resides on the cytoplasmic side of membrane microdomains (Bickel et al, 1997; Carcea et al, 2010). Flotillin-2 labeling showed a different distribution pattern on each substrate (Fig. 5M-O). Following exposure to ethanol, there was an insignificant decrease in Flotillin-2 labeling intensity on L1CAM (Fig. 5M), an insignificant increase on PLL (Fig. 5N) and a significant increase on Ncad (Fig. 5O).

Collectively the data indicate that markers for membrane microdomains can be altered by ethanol exposure, a finding that is consistent with previous work in other cell types (e.g. Blanco et al, 2008; Dolganiac et al, 2006; Nourissat et al, 2008), but their responses show no consistent correlation with the functional rescue we observe in growth cones extending on L1CAM. Only the distribution of L1CAM itself correlates with functional rescue.

Discussion

We find that ethanol levels commonly observed in social drinking can render cortical axonal growth cones unresponsive to Sema3A, LPA and Netrin. This effect is dependent on context; neurons grown on a substrate of L1CAM can resist the silencing effects of ethanol at concentrations that approach physiologically observed maxima, while those on Ncad or PLL cannot. Collectively, the data support a model in which axonal responses to guidance cues will be differentially sensitive to ethanol exposure depending on the environment in which they are growing.

Ethanol inhibits axonal growth cone responses to several guidance cues and our data support that growth cones are the targets. Experiments testing growth cone recovery following collapse suggest that neither cues nor ethanol detectably altered the substrates that were used. It remains possible that the different substrates could have also altered the sensitivity to particular cues, but it is unlikely that this would greatly alter the interpretation since the concentrations of the cues that were used was sufficient to induce collapse in the absence of ethanol and low enough to permit recovery.

Since inhibition of growth cone collapse was common to several experimental conditions, ethanol most likely disrupts signaling element(s) held in common. For Sema3A, Netrin and LPA, it has been shown that their respective receptors are recruited to lipid raft enriched membrane microdomains following ligand binding and that this redistribution is required to

generate appropriate responses to Sema3A and Netrin (Guirland et al, 2004), and to LPA (Peres et al, 2003). Ethanol intercalates into lipid bilayers (Laev et al, 1996; Mrak, 1992) where, for example, it can prevent pathogen-stimulated recruitment of Toll-like receptor-4 into lipid rafts and the initiation of subsequent signaling pathways in macrophages and monocytes (Dai et al, 2005; Dai and Pruett, 2006; Dolganiuc et al, 2006; Szabo et al, 2007). Thus, it is plausible that ethanol adopts a similar mechanism in growth cones by either altering the steady state organization of the plasma membrane or by preventing the membrane reorganization required to generate appropriate responses. Consistent with this idea, we find that axonal growth cones on an L1CAM substrate maintain normal surface intensity distributions of L1CAM following exposure to ethanol, but notably, the distributions of membrane microdomain markers, CtxB and Flotillin-2, were not similarly preserved, suggesting that the positive effect of L1CAM substrates will be limited.

Alternatively, or as a consequence of disrupted membrane microdomains, it is possible that ethanol inhibits the actin reorganization required to produce growth cone collapse. Sema3A, Netrin, and LPA each activate distinct signaling pathways (Campbell and Holt, 2003; Huber et al, 2003), but under the conditions examined, all converge on alterations to the actin cytoskeleton that can produce growth cone collapse (Gallo and Letourneau, 2004). Ethanol can inhibit actin polymerization in macrophages (Dai and Pruett, 2006) or induce actin depolymerization in cerebellar granule cells (Offenhauser et al, 2006). Mechanisms that lead to cofilin inactivation or increased RhoGTPase activity can increase resistance to these effects of ethanol on actin (Offenhauser et al, 2006; Rothenfluh et al, 2006), but it is not known whether these pathways are direct targets for ethanol.

Some insight into the mechanism of ethanol action in cortical axons may be provided by our data, which show that neurons growing on an L1CAM substrate are resistant to ethanol-mediated silencing of growth cone responses to guidance cues. L1CAM is highly abundant and natively distributed in both raft and non-raft domains (Kamiguchi et al, 2000; IC and DLB, unpublished observations), and can be stably anchored to actin via ankyrin (Bennett and Lambert, 1999; Gil et al, 2003). Such anchored transmembrane proteins have been proposed to stabilize and organize plasma membrane by generating compartment boundaries and by limiting protein and lipid diffusion (Kusumi et al, 2005). An L1CAM substrate would also be anticipated to bind heterophilically to other members of the L1CAM family found in cortical neurons: TAG1, a GPI-linked family member enriched in lipid rafts (Furley et al, 1990), as well as CHL1 (Demyanenko et al, 2010; Maness and Schachner, 2007). That an L1CAM substrate may help to stabilize or protect the plasmalemma from the effects of ethanol is also consistent with previous work in neurons following a sustained, 48 h, exposure to ethanol. In cortical neurons L1CAM localization and internalization remain unchanged relative to controls in growth cones extending on L1CAM substrates (Hoffman et al, 2008) and cerebellar granule neurons growing on a substrate of L1CAM are resistant to ethanol-mediated neuronal apoptosis (Gubitosi-Klug et al, 2007). L1CAM can also have dynamic interactions with actin via its binding to ERM proteins (Ezrin, Radixin, Moesin) (Dickson et al, 2008). ERMs can be regulated by guidance cues (Mintz et al, 2008) and in immune cells, ERMs play integral roles in both migration and cell-cell recognition by coordinating actin cytoskeletal dynamics and interactions with key transmembrane partners as they enter or exit membrane rafts (Viola et al, 2007). In this light it will be important in future experiments to determine whether the protective effects of L1CAM seen in the presence of ethanol will extend to cortical neuron migration.

Whether or not the principal effects of an L1CAM substrate are on membrane integrity, actin cytoskeleton, or signaling, it is clear that the effects of acute ethanol exposure on cortical neurons do not fit a model in which ethanol binds and inhibits L1CAM adhesion. In certain cell lines acute exposure to ethanol can reduce L1CAM mediated adhesion (Charness

et al, 1994, Ramanathan et al, 1996, Wilkemeyer and Charness, 1998), but similar exposures have no effect on L1CAM adhesion in cerebellar granule cells (Bearer et al, 1999) or in many cell lines (Vallejo et al, 1997; Wilkemeyer and Charness, 1998). Thus, the actions of ethanol and its interactions with L1CAM cannot be readily generalized across cell types reflecting cell type-specific differences in membrane composition as well as the complexity of L1CAM function.

A broad array of evidence in humans and in animal models supports that ethanol interferes with neuronal maturation (Miranda et al, 2008). Additional evidence supports that particular neuron populations or stages of development are selectively vulnerable to ethanol exposure (e.g. Cheema et al, 2000; Luo and Miller, 1998; West and Hamre, 1985). Taken in this context, our findings suggest that axonal guidance will be more or less vulnerable to the effects of ethanol depending on the environment through which axons are growing. Additionally, our data indicate that a brief period of exposure (1 h) can render growth cones insensitive to cues. Since several lines of investigation in humans and animal models emphasize that a brief, binge-like exposure to ethanol is particularly devastating (e.g. Bailey et al, 2004; Boehm et al, 2008; Jacobsen et al, 1998; Pierce and West, 1986; Streisguth et al, 1990), it will be important to determine whether diminished growth cone responses are restricted to short periods or whether growth cones can recover responsiveness following sustained periods of ethanol exposure.

Acknowledgments

This work was supported by NIH grants NIAAA R01AA01497 and NINDS NS050634 and NIH training grant 5T32MH087004. We thank ChunYing Ko and Roxana Mesias for their excellent technical assistance. Confocal laser scanning microscopy was performed in part at the MSSM-Microscopy Shared Resource Facility, supported with funding from NIH-NCI shared resources grant (5R24 CA095823-04), NSF Major Research Instrumentation grant (DBI-9724504) and NIH shared instrumentation grant (1 S10 RR0 9145-01).

References

- Arevalo E, Shanmugasundararaj S, Wilkemeyer MF, Dou X, Chen S, Charness ME, Miller KW. An alcohol binding site on the neural cell adhesion molecule L1CAM. *Proc Natl Acad Sci USA*. 2008; 105:371–375. [PubMed: 18165316]
- Bailey BN, Delaney-Black V, Covington CY, Ager J, Janisse J, Hannigan JH, Sokol RJ. Prenatal exposure to binge drinking and cognitive and behavioral outcomes at age 7 years. *Am J Obstet Gynecol*. 2004; 191:1037–1043. [PubMed: 15467586]
- Bearer CF, Swick AR, O'Riordan MA, Cheng G. Ethanol inhibits L1CAM-mediated neurite outgrowth in postnatal rat cerebellar granule cells. *J Biol Chem*. 1999; 274:13264–13270. [PubMed: 10224086]
- Bearer CF. L1CAM cell adhesion molecule signal cascades: targets for ethanol developmental neurotoxicity. *Neurotoxicology*. 2001; 22:625–633. [PubMed: 11770884]
- Bennett V, Lambert S. Physiological roles of axonal ankyrins in survival of premyelinated axons and localization of voltage-gated sodium channels. *J Neurocytol*. 1999; 28:303–318. [PubMed: 10739573]
- Bentley D, Toroian-Raymond A. Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature*. 1986; 323:712–5. [PubMed: 3773996]
- Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *J Biol Chem*. 1997; 272:13793–13802. [PubMed: 9153235]
- 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]

- Boehm SL 2nd, Moore EM, Walsh CD, Gross CD, Cavelli AM, Gigante E, Linsenbardt DN. Using drinking in the dark to model prenatal binge-like exposure to ethanol in C57BL/6J mice. *Dev Psychobiol.* 2008; 50:566–578. [PubMed: 18683190]
- Bovolenta P, Mason C. Growth cone morphology varies with position in the developing mouse visual pathway from retina to first targets. *J Neurosci.* 1987; 7:1447–1460. [PubMed: 3572487]
- Brick J. Standardization of alcohol calculations in research. *Alcohol Clin Exp Res.* 2006; 30:1276–1287. [PubMed: 16899030]
- Campbell DS, Holt CE. Apoptotic pathway and MAPKs differentially regulate chemotropic responses of retinal growth cones. *Neuron.* 2003; 27:939–952. [PubMed: 12670423]
- Carcea I, Ma'ayan A, Mesias R, Sepulveda B, Salton SRJ, Benson DL. Flotillin-mediated endocytic events dictate cell-type specific responses to Semaphorin 3A. *J Neurosci.* 2010; 30:15317–15329. [PubMed: 21068336]
- Castellani V, Chedotal A, Schachner M, Favire-Sarrailh C, Rougon G. Analysis of the L1CAM-deficient mouse phenotype reveals cross-talk between sema3A and L1CAM signaling pathways in axonal guidance. *Neuron.* 2000; 27:237–249. [PubMed: 10985345]
- Castellani V, De Angelis E, Kenwrick S, Rougon G. Cis and trans interactions of L1CAM with neuropilin-1 control axonal responses to semaphorin3A. *EMBO J.* 2002; 23:6348–6357. [PubMed: 12456642]
- Chappell TD, Margret CP, Li CX, Waters RS. Long-term effects of prenatal alcohol exposure on the size of the whisker representation in juvenile and adult rat barrel cortex. *Alcohol.* 2007; 41:239–251. [PubMed: 17630085]
- Charness ME, Safran RM, Perides G. Ethanol inhibits neural cell-cell adhesion. *J Biol Chem.* 1994; 269:9304–9309. [PubMed: 8132668]
- Cheema ZF, West JR, Miranda RC. Ethanol induces Fas/Apo [apoptosis]-1 mRNA and cell suicide in the developing cerebral cortex. *Alcohol Clin Exp Res.* 2000; 24:535–543. [PubMed: 10798591]
- Chen Y, Stevens B, Chang J, Milbrandt J, Barres BA, Hell JW. NS21: re-defined and modified supplement B27 for neuronal cultures. *J Neurosci Methods.* 2008; 171:239–247. [PubMed: 18471889]
- Chien CB, Cornel EM, Holt CE. Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. *Neuron.* 1993; 11:237–251. [PubMed: 8352941]
- Cheng L, Lemmon V. Pathological missense mutations of neural cell adhesion molecule L1 affect neurite outgrowth and branching on an L1 substrate. *Mol Cell Neurosci.* 2004; 27:522–530. [PubMed: 15555929]
- Clamp PA, Lindsley TA. Early events in the development of neuronal polarity in vitro are altered by ethanol. *Alcohol Clin Exp Res.* 1998; 22:1277–1284. [PubMed: 9756043]
- Cuzon VC, Yeh PW, Yanagawa Y, Obata K, Yeh HH. Ethanol consumption during early pregnancy alters the disposition of tangentially migrating GABAergic interneurons in the fetal cortex. *J Neurosci.* 2008; 28:1854–1864. [PubMed: 18287502]
- Dai Q, Zhang J, Pruett SB. Ethanol alters cellular activation and CD14 partitioning in lipid rafts. *Biochem Biophys Res Commun.* 2005; 332:37–42. [PubMed: 15896296]
- Dai Q, Pruett SB. Ethanol suppresses LPS-induced Toll-like receptor 4 clustering, reorganization of the actin cytoskeleton, and associated TNF-alpha production. *Alcohol Clin Exp Res.* 2006; 30:1436–1444. [PubMed: 16899048]
- Demyanenko GP, Siesser PF, Wright AG, Brennaman LH, Bartsch U, Schachner M, Maness PF. L1 and CHL1 cooperate in thalamocortical axon targeting. *Cereb Cortex.* Epub June. 2010; 24:2010.
- Dent EW, Barnes AM, Tang F, Kalil K. Netrin-1 and semaphorin3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. *J Neurosci.* 2004; 24:3002–3012. [PubMed: 15044539]
- Dickson TC, Mintz CD, Benson DL, Salton SRJ. Functional binding interaction identified between the axonal CAM L1CAM and members of the ERM family. *J Cell Biol.* 2002; 157:1105–1112. [PubMed: 12070130]
- Dolganic A, Bakis G, Kodys K, Mandrekar P, Szabo G. Acute ethanol treatment modulates Toll-like receptor-4 association with lipid rafts. *Alcohol Clin Exp Res.* 2006; 30:76–85. [PubMed: 16433734]

- Fransen E, Lemmon V, Van Camp G, Vits L, Coucke P, Willems PJ. CRASH syndrome: clinical spectrum of corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraparesis and hydrocephalus due to mutations of one single gene, L1. *Eur J Hum Genet.* 1995; 3:273–284. [PubMed: 8556302]
- Fransen E, D'Hooge R, Van Camp G, Verhoye M, Sijbers J, Reyniers E, Soriano P, Kamiguchi H, Willemsen R, Koekkoek SK, 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. *Hum Mol Genet.* 1998; 7:999–1009. [PubMed: 9580664]
- Fryer SL, Schweinsburg BC, Bjorkquist OA, Frank LR, Mattson SN, Spadoni AD, Riley EP. Characterization of white matter microstructure in fetal alcohol spectrum disorders. *Alcohol Clin Exp Res.* 2009; 33:514–521. [PubMed: 19120066]
- Fukushima N, Ye X, Chun J. Neurobiology of lysophosphatidic acid signaling. *Neuroscientist.* 2002; 8:540–550. [PubMed: 12467376]
- Furley AJ, Morton SB, Manalo D, Karageorgos D, Dodd J, Jessell TM. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell.* 1990; 61:157–170. [PubMed: 2317872]
- Gallo G, Letourneau PC. Regulation of growth cone actin filaments by guidance cues. *J Neurobiol.* 2004; 58:92–102. [PubMed: 14598373]
- Gil OD, Sakurai T, Bradley AE, Fink MY, Cassella MR, Kuo JA, Felsenfeld DP. Ankyrin binding mediates L1CAM interactions with static components of the cytoskeleton and inhibits retrograde movement of L1CAM on the cell surface. *J Cell Biol.* 2003; 162:719–730. [PubMed: 12925712]
- Goslin K, Banker G. Experimental observations on the development of polarity by hippocampal neurons in culture. *J Cell Bio.* 1989; 108:1507–1516. [PubMed: 2925793]
- Gubitosi-Klug R, Larimer CG, Bearer CF. L1 cell adhesion molecule is neuroprotective of alcohol induced cell death. *Neurotoxicology.* 2007; 28:457–462. [PubMed: 17267039]
- Guirland C, Suzuki S, Kojima M, Lu B, Zheng JQ. Lipid rafts mediate chemotropic guidance of nerve growth cones. *Neuron.* 2004; 42:51–62. [PubMed: 15066264]
- Hannigan JH, O'Leary-Moore SK, Berman RF. Postnatal environment or experimental amelioration of neurobehavioral effects of perinatal alcohol exposure in rats. *Neurosci Biobehav Rev.* 2007; 31:202–211. [PubMed: 16911827]
- Hoffman EJ, Mintz CD, Wang S, McNickle DG, Salton SRJ, Benson DL. Effects of ethanol on axon outgrowth and branching in developing rat cortical neurons. *Neurosci.* 2008; 157:556–565.
- Hopker VH, Shewan D, Tessier-Lavigne M, Poo M, Holt C. Growth cone attraction to netrin-1 is converted to repulsion by laminin-1. *Nature.* 1999; 401:69–73. [PubMed: 10485706]
- Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annu Rev Neurosci.* 2003; 26:509–563. [PubMed: 12677003]
- Jacobson JL, Jacobson SW, Sokol RJ, Ager JW Jr. Relation of maternal age and pattern of pregnancy drinking to functionally significant cognitive deficit in infancy. *Alcohol Clin Exp Res.* 1998; 22:345–351. [PubMed: 9581639]
- Jalink K, Hordijk PL, Moolenaar WH. Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. *Biochim Biophys Acta.* 1994; 1198:185–196. [PubMed: 7819274]
- Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. *Lancet.* 1973; 2:999–1001. [PubMed: 4127281]
- Jouet M, Rosenthal A, Armstrong G, MacFarlane J, Stevenson R, Paterson J, Metzenberg A, Ionasescu V, Temple K, Kenwrick S. X-linked spastic paraplegia (SPG1), MASA syndrome and X-linked hydrocephalus result from mutations of the L1 gene. *Nat Genet.* 1994; 7:402–407. [PubMed: 7920659]
- Kamiguchi H, Lemmon V. Recycling of the cell adhesion molecule L1CAM in axonal growth cones. *J Neurosci.* 2000; 20:3676–3686. [PubMed: 10804209]
- Kapfhammer JP, Xu H, Raper JA. The detection and quantification of growth cone collapsing activities. *Nature Prot.* 2007; 2:2005–2011.

- Kusumi A, Ike H, Nakada C, Murase K, Fujiwara T. Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules. *Semin Immunol.* 2005; 17:3–21. [PubMed: 15582485]
- Laev H, Hungund BL, Karpiak SE. Cortical cell plasma membrane alterations after in vitro alcohol exposure: prevention by GM1 ganglioside. *Alcohol.* 1996; 13:187–194. [PubMed: 8814655]
- Lebel C, Rasmussen C, Wyper K, Walker L, Andrew G, Yager J, Beaulieu C. Brain diffusion abnormalities in children with fetal alcohol spectrum disorder. *Alcohol Clin Exp Res.* 2008; 32:1732–1740. [PubMed: 18671811]
- Li L, Coles CD, Lynch ME, Hu X. Voxelwise and skeleton-based region of interest analysis of fetal alcohol syndrome and fetal alcohol spectrum disorders in young adults. *Hum Brain Mapp.* 2009; 30:3265–3274. [PubMed: 19278010]
- Lindsley TA, Kerlin AM, Rising LJ. Time-lapse analysis of ethanol's effects on axon growth in vitro. *Brain Res Dev Brain Res.* 2003; 147:191–199.
- Lindsley TA, Miller MW, Littner Y, Bearer CF. Signaling pathways regulating cell motility: a role in ethanol teratogenicity? *Alcohol Clin Exp Res.* 2006; 30:1445–1450. [PubMed: 16899049]
- Livy DJ, Miller EK, Maier SE, West JR. Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicol Teratol.* 2003; 25:447–458. [PubMed: 12798962]
- Lugo JN, Marino MD, Cronise K, Kelly SJ. Effects of alcohol exposure during development on social behavior in rats. *Physiol Behav.* 2003; 78:185–194. [PubMed: 12576115]
- Luo J, Miller MW. Growth factor-mediated neural proliferation: target of ethanol toxicity. *Brain Res Brain Res Rev.* 1998; 27:157–167. [PubMed: 9622617]
- Ma X, Coles CD, Lynch ME, Laconte SM, Zurkiya O, Wang D, Hu X. Evaluation of corpus callosum anisotropy in young adults with fetal alcohol syndrome according to diffusion tensor imaging. *Alcohol Clin Res Exp.* 2005; 29:1214–1222.
- Maier SE, Chen WJ, Miller JA, West JR. Fetal alcohol exposure and temporal vulnerability regional differences in alcohol-induced microencephaly as a function of the timing of binge-like alcohol exposure during rat brain development. *Alcohol Clin Res Exp.* 1997; 21:1418–1428.
- 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]
- Margret CP, Li CX, Elberger AJ, Matta SG, Chappell TD, Waters RS. Prenatal alcohol exposure alters the size, but not the pattern, of the whisker representation in neonatal rat barrel cortex. *Exp Brain Res.* 2005; 165:167–178. [PubMed: 15856205]
- Margret CP, Li CX, Chappell TD, Elberger AJ, Matta SG, Waters RS. Prenatal alcohol exposure delays the development of the cortical barrel field in neonatal rats. *Exp Brain Res.* 2006a; 172:1–13. [PubMed: 16506013]
- Margret CP, Chappell TD, Li CX, Jan TA, Matta SG, Elberger AJ, Waters RS. Prenatal alcohol exposure (PAE) reduces the size of the forepaw representation in forepaw barrel subfield (FBS) cortex in neonatal rats: relationship between periphery and central representation. *Exp Brain Res.* 2006b; 172:387–396. [PubMed: 16424976]
- Miller MW. Fetal alcohol effects on the generation and migration of cerebral cortical neurons. *Science.* 1986; 231:301–311.
- Miller MW, al-Rabia S. Effects of prenatal exposure to ethanol on the number of axons in the pyramidal tract of the rat. *Alcohol Clin Exp Res.* 1994; 18:346–354.
- Miñana R, Climent E, Baretino D, Segui JM, Renau-Piqueras J, Guerri C. Alcohol exposure alters the expression pattern of neural cell adhesion molecules during brain development. *J Neurochem.* 2000; 75:954–964. [PubMed: 10936176]
- Mintz CD, Carcea I, McNickle DG, Dickson TC, Ge Y, Salton SR, Benson DL. ERM proteins regulate growth cone responses to Sema3A. *J Comp Neurol.* 2008; 510:351–366. [PubMed: 18651636]
- Miranda, RC.; Santillano, DR.; Camarillo, C.; Dohrman, D. Nagy, LE., editor. Modeling the impact of alcohol on cortical development in a dish: strategies from mapping neural stem cell fate.; *Alcohol: methods and protocols, Methods in molecular biology.* 2008. p. 151-168.

- Mrak RE. Opposite effects of dimethyl sulfoxide and ethanol on synaptic membrane fluidity. *Alcohol*. 1992; 9:513–517. [PubMed: 1472307]
- Nourissat P, Travert M, Chevanne M, Tekpli X, Rebillard A, Le Moigne-Müller G, Rissel M, Cillard J, Dimanche-Boitrel M-T, Lagadic-Gossmann D, Sergeant O. Ethanol induces oxidative stress in primary rat hepatocytes through the early involvement of lipid raft clustering. *Hepatology*. 2008; 47:59–70. [PubMed: 18038449]
- Offenhauser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, Rossi P, D'Angelo E, Frassoni C, Amadeo A, Tocchetti A, Pozzi B, Disanza A, Guarnieri D, Betsholtz C, Scita G, Heberlein U, Di Fiore PP. Increased ethanol resistance and consumption in Eps8 knockout mice correlates with altered actin dynamics. *Cell*. 2006; 127:213–226. [PubMed: 17018287]
- Paratcha G, Ledda F, Ibañez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell*. 2003; 113:867–879. [PubMed: 12837245]
- Peres C, Yart A, Perret B, Salles JP, Raynal P. Modulation of phosphoinositide 3-kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling. *FEBS Lett*. 2003; 534:164–168. [PubMed: 12527380]
- Pierce DR, West JR. Blood alcohol concentration: A critical factor for producing fetal alcohol effects. *Alcohol*. 1986; 3:269–272. [PubMed: 3638973]
- Ramanathan R, Wilkemeyer MF, Mittal B, Perides G, Charness ME. Alcohol inhibits cell-cell adhesion mediated by human L1CAM. *J Cell Biol*. 1996; 133:381–390. [PubMed: 8609170]
- Rothenfluh A, Threlkeld RJ, Bainton RJ, Tsai LT, Lasek AW, Heberlein U. Distinct behavioral responses to ethanol are regulated by alternate RhoGAP18B isoforms. *Cell*. 2006; 127:199–211. [PubMed: 17018286]
- Siegenthaler JA, Miller MW. Transforming growth factor beta1 modulates cell migration in rat cortex: effects of ethanol. *Cereb Cortex*. 2004; 14:791–802. [PubMed: 15084492]
- Sowell ER, Johnson A, Kan E, Lu LH, Van Horn JD, Toga AW, O'Connor MJ, Bookheimer SY. Mapping white matter integrity and neurobehavioral correlates in children with fetal alcohol spectrum disorders. *J Neurosci*. 2008; 28:1313–1319. [PubMed: 18256251]
- Streissguth AP, Barr HM, Sampson PD. Moderate prenatal alcohol exposure: Effects on child IQ and learning problems at age 7.5 years. *Alcohol Clin Exp Res*. 1990; 14:662–669. [PubMed: 2264594]
- 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, He M, O'Riordan MA, Farkas C, Buck K, Lemmon V, Bearer CF. Ethanol inhibits L1CAM cell adhesion molecule activation of mitogen-activated protein kinases. *J Neurochem*. 2006; 96:1480–1490. [PubMed: 16478533]
- Tran TD, Kelly SJ. Critical periods for ethanol-induced cell loss in the hippocampal formation. *Neurotoxicol Teratol*. 2003; 25:519–528. [PubMed: 12972065]
- Vallejo Y, Hortsch M, Dubreuil RR. Ethanol does not inhibit the adhesive activity of Drosophila Neuroglial or human L1 in Drosophila S2 tissue culture cells. *J Biol Chem*. 1997; 272:12244–12247. [PubMed: 9115300]
- Viola A, Gupta N. Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. *Nat Rev Immunol*. 2007; 7:889–896. [PubMed: 17948020]
- Watanabe H, Yamazaki 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. *J Neurochem*. 2004; 89:142–151. [PubMed: 15030398]
- West JR, Hamre KM. Effects of alcohol exposure during different periods of development: changes in hippocampal mossy fibers. *Brain Res*. 1985; 349:280–284. [PubMed: 3986593]
- West JR, Hodges CA, Black AC Jr. Prenatal exposure to ethanol alters the organization of hippocampal mossy fibers in rats. *Science*. 1981; 211:957–959. [PubMed: 7466371]
- Wilkemeyer MF, Charness ME. Characterization of ethanol-sensitive and insensitive fibroblast cell lines expressing human L1. *J Neurochem*. 1998; 71:2382–2391. [PubMed: 9832136]
- Wilkemeyer MF, Sebastian AB, Smith SA, Charness ME. Antagonists of alcohol inhibition of cell adhesion. *Proc Natl Acad Sci USA*. 2000; 97:3690–3695. [PubMed: 10725368]

- Wong EV, Kenwrick S, Willems P, Lemmon V. Mutations in the cell adhesion molecule L1 cause mental retardation. *Trends Neurosci.* 1995; 18:168–172. [PubMed: 7778187]
- Wozniak JR, Mueller BA, Chang PN, Muetzel RL, Caros L, Lim KO. Diffusion tensor imaging in children with fetal alcohol spectrum disorders. *Alcohol Clin Res Exp.* 2006; 30:1799–1806.
- Wozniak JR, Muetzel RL, Mueller BA, McGee CL, Freerks MA, Ward EE, Nelson ML, Chang PN, Lim KO. Microstructural corpus callosum anomalies in children with prenatal alcohol exposure: an extension of previous diffusion tensor imaging findings. *Alcohol Clin Res Exp.* 2009; 33:1825–1835.

Figure 1. Ethanol prevents growth cone collapse in response to Sema3A on PLL and Ncad, but response is restored on L1CAM

Inverted images of fluorescent photomicrographs show collapse response in cortical neurons. Neurons plated on PLL were exposed to control media (A) or Sema3A (B) and then fixed, and F-actin was labeled with Rhodamine-phalloidin. Graph in (C) shows the effect of ethanol (x-axis) on Sema3A mediated collapse. Neurons were plated on PLL and exposed to the [EtOH] indicated on horizontal axis for 60 minutes followed by control media (gray, open triangles) or 100ng/ml Sema3A (black, closed triangles). Ethanol significantly inhibits Sema3A-mediated growth cone collapse while ethanol alone has no effect ($n = 2$ coverslips/condition; 3 separate cultures; 1218 growth cones were analyzed on

average per condition). 2-way ANOVA indicates the effect of substrate and ethanol are significant (** $p < 0.0001$). Bonferroni post tests show significant difference with respect to control media conditions at the same ethanol concentration (* $p < 0.05$). **(D)** Graph compares growth cone collapse in response to Sema3A on L1CAM, PLL and Ncad substrates. 2-way ANOVA indicates the effect of substrate and ethanol are significant (** $p < 0.0001$). Bonferroni post tests indicate a significant decrease in collapse on PLL at all concentrations of ethanol tested (* $p < 0.05$ for 12.5 mM, ** $p < 0.001$ for 25 and 50 mM; 2-way ANOVA) and on Ncad at 50 mM ethanol (†† $p < 0.001$). At least 4 coverslips per condition (4 - 12), from 2 separate cultures; at least 500 growth cones per condition for Ncad and L1CAM substrates.

Figure 2. Ethanol generally inhibits cue-induced growth cone collapse and L1CAM generally rescues growth cone responses

(A) Bar graph illustrates ethanol-mediated inhibition of Netrin-mediated growth cone collapse on laminin. At 0mM ethanol, axonal growth cones collapse in response to Netrin in neurons plated on laminin, but not on PLL (gray vs. hatched and white bars, respectively). At all concentrations of ethanol tested, growth cone collapse on laminin in response to Netrin is significantly inhibited (one way ANOVA ($p=0.0008$) and Tukey's post tests $\dagger p<0.05$, $*p<0.01$; at least 467 growth cones were analyzed per condition; 2 coverslips/condition; 3 separate cultures). (B) Ethanol also inhibits growth cone collapse in response to LPA in neurons growing on PLL (gray triangles), or Ncad (gray circles) ($*p<0.05$; $**p<0.001$). Similar to our findings with Sema3A, the collapse response is retained in neurons grown on L1CAM (black triangles). 2-way ANOVA shows significant differences with substrate and ethanol ($p<0.0001$). Bonferroni post test indicates differences with respect to L1CAM, $*p<0.01$, $p<0.001$. At least 6 coverslips per condition (6-8) from 3 separate cultures; at least 898 growth cones per condition.

Figure 3. Ethanol modestly impairs growth cone extension

Graphs summarize the quantitative analysis of axon outgrowth observed over 17 hours in the presence or absence of 25 mM ethanol beginning 3h after addition of ethanol. For each parameter, the control and ethanol groups were compared in paired, two tailed, t-tests. **(A)** Axonal outgrowth velocity was significantly decreased in the ethanol group, and this is reflected in a modest, but significant reduction in **(B)** cumulative growth. The effect on **(C)** displacement (vector distance) is of a greater magnitude. Even beginning at 1h following ethanol exposure, the same three parameters are significantly decreased in neurons exposed to 25 mM ethanol compared to control: **(D)** velocity (* $p < 0.05$), **(E)** cumulative growth and **(F)** displacement. **A-C**, $n > 43$ neurons per group, **D-F**, $n > 20$; (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Figure 4. Ethanol mediated changes in L1CAM and CtxB distribution in growth cones

Proximal-to-distal distribution of immunolabeling for L1CAM (ASCS4) or cholera toxin B (CtxB) on the substrates shown following exposure to 0mM or 50mM EtOH. Each point is the mean intensity ($n = 30$) at sites along the length of growth cones with proximal at left and distal to the right. In order to compare growth cones of different lengths, measurements were normalized. While L1CAM binding and CtxB show distinct proximal-distal gradients, the effect of ethanol shifts the entire curve up or down, and does not seem to mediate its effects differentially with respect to growth cone regions. Similar data were observed with Flotillin-2 labeling (not shown). au = arbitrary units. Symbols are defined in legends for each graph.

Figure 5. Ethanol alters the distribution of L1CAM in growth cones on PLL and Ncad, but not on L1CAM

(A-I) Neurons exposed to 0 or 50mM EtOH were immunolabeled for surface L1CAM (B, C, E, F, H, I) and the resulting fluorescence intensity was quantified (see Methods) and compared in cumulative histograms (A,D,G). On an L1CAM substrate, L1CAM intensity is distributed similarly quantitatively and qualitatively in control growth cones (0mM ethanol; solid line) and those exposed to 50 mM ethanol (dotted line). On PLL (D-F), there is an increase in L1CAM intensity in growth cones exposed to 50mM ethanol compared to control (* $p < 0.05$) (G-I) On Ncad substrate, 50mM ethanol also increases L1CAM labeling intensity (Kruskal-Wallis test comparing L1CAM labeling on all 3 substrates, $p < 0.0001$;

Dunn's post test, $*p < 0.05$, $**p < 0.01$). On **(J)** L1CAM and **(L)** Ncad, there is a shift of CtxB labeling towards higher intensities and on **(K)** PLL toward lower intensities following exposure to 50mM EtOH (Kruskal-Wallis test, $p < 0.0001$; Dunn's post test, $**p < 0.001$ for L1CAM and Ncad). Neurons labeled for Flotillin-2 on **(M)** L1CAM show an insignificant shift towards lower intensities in ethanol-treated neurons compared to control, and on **(N)** PLL, there was little change. **(O)** On Ncad, the increase toward higher intensities was significant (Kruskal-Wallis test, $p < 0.0001$; Dunn's post test $**p < 0.01$). au = arbitrary units. For **A, D, G** and **J-O**, at least 10 neurons per group; 29 measurements per neuron.