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Ethanol Alters BDNF-induced Rho GTPase Activation in Axonal Growth Cones

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Abstract

Background—The effects of ethanol on development of postmitotic neurons include altered neurite outgrowth and differentiation, which may contribute to neuropathology associated with fetal alcohol spectrum disorders. We previously reported that ethanol exposure alters axon growth dynamics in dissociated cultures of rat hippocampal pyramidal neurons. Given the important regulatory role of small Rho GTPases in cytoskeletal reorganization associated with axon growth, and reports that ethanol alters whole cell Rho GTPase activity in other cell types, this study explored the hypothesis that ethanol alters Rho GTPase activity specifically in axonal growth cones.

Methods—Fetal rat hippocampal pyramidal neurons were maintained in dissociated cultures for 1 day in control medium or medium containing 11–43 mM ethanol. Some cultures were also treated with brain-derived neurotrophic factor (BDNF), an activator of Rac1 and Cdc42 GTPases that promotes axon extension. Levels of active Rho GTPases in growth cones were measured using in situ binding assays for GTP-bound Rac1, Cdc42 and RhoA. Axon length, growth cone area and growth cone surface expression of TrkB, the receptor for BDNF, were assessed by digital morphometry and immunocytochemistry.

Results—Although ethanol increased the surface area of growth cones, the levels of active Rho GTPases in axonal growth cones were not affected in the absence of exogenous BDNF. In contrast, ethanol exposure inhibited BDNF-induced Rac1/Cdc42 activation in a dose-dependent manner and increased RhoA activation at the highest concentration tested. Similar TrkB expression was observed on the surface of axonal growth cones of control and ethanol-treated neurons.

Conclusions—These results reveal an inhibitory effect of ethanol on growth cone signaling via small Rho GTPases during early stages of hippocampal development in vitro, and suggest a mechanism whereby ethanol may disrupt neurotrophic factor regulation of axon growth and guidance.

Keywords

neurite outgrowth; Rho GTPases; BDNF; growth cone; hippocampal cultures

Introduction

Fetal alcohol spectrum disorder (FASD) is associated with neurological and behavioral problems believed to result from the specific action of alcohol on key regulators of neurodevelopmental events (Goodlett et al., 2005; Harris et al., 2008; Morrow et al., 2004).

A consistent histopathologic feature of FASD in humans and animal models is abnormal axonal and dendritic morphology in various brain regions (Lindsley, 2006; Pentney and Miller, 1992). The hippocampus is especially vulnerable to damage by ethanol during its development, where stunted dendritic arbors and abnormal axon trajectories and synaptic connections are thought to contribute to the cognitive deficits associated with FASD (Berman and Hannigan, 2000; Hamilton et al., 2003; West et al., 1981).

For axons and dendrites to acquire their characteristic shapes and connectivity, they must constantly respond to extracellular signals that reorganize actin and microtubules in their growth cones. The Rho family guanosine triphosphatases (GTPases), play important roles in this signaling by acting as “molecular switches” linking upstream growth and guidance signals with downstream effectors that regulate the cytoskeleton, as well as membrane trafficking and transcriptional activation (reviewed in Govek et al., 2005; Lowery and Van Vactor, 2009). In general, Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (cell division cycle 42) are positive regulators that enhance axon growth and the formation of veil-like lamellipodia and finger-like filopodia in growth cones, whereas RhoA (Ras homologous member A) is a negative regulator that induces growth cone collapse and axon retraction. Upstream, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) act on Rho GTPases to positively and negatively regulate their activity, respectively. Downstream, important effectors for active (GTP-bound) Rac1 and Cdc42, are the p21-activated kinases (PAKs). An important downstream effector for GTP-bound RhoA is Rho-kinase/ROCK.

The potential importance of identifying Rho GTPase signaling as vulnerable to ethanol during development is highlighted by increasing evidence that mutations in components of Rho signaling pathways are associated with neurodevelopmental diseases characterized by cognitive deficits. For example, nonsyndromic X-linked mental retardation (MRX) is linked to mutations in any of several loci on the X chromosome that alters the activity of a GTPase-activating protein (GAP), guanine nucleotide exchange factor (GEF) or p21-activated kinase 3 (PAK3) downstream effector of Rac1/Cdc42 (reviewed in Kasri and Van Aelst, 2008).

Some studies using non-neuronal cells have shown that ethanol-induced alterations in F-actin organization are mediated by small Rho GTPases regulating the dynamics of the actin cytoskeleton (Guasch et al., 2003; Qian et al., 2003; Schaffert et al., 2006). However, only a single study to date has examined whether altered Rho GTPase signaling, measured in whole cell lysates, may contribute to impaired process outgrowth in developing neurons (Joshi et al., 2006). To our knowledge no studies have investigated whether ethanol affects the local activation of Rho GTPases within neuronal growth cones. This is important because the growth cone, which accounts for no more than 5-10 percent of the neuron, is the highly localized site for detecting and responding to growth regulating extracellular signals. In addition, the activity of different Rho GTPases in the growth cone is regulated by their specific subcellular localization (Aoki et al., 2004; Pertz et al., 2008; Picard et al., 2009), relationships that are disrupted by lysis in whole cell assays.

In the present study, we used in situ binding assays to assess Rac1 and Cdc42 activity, and RhoA activity, in axonal growth cones of embryonic rat hippocampal pyramidal neurons maintained in vitro for 1 day in control medium or medium containing 11-87 mM ethanol (to approximate concentrations of 50-400mg/dl in blood). We have previously reported that these conditions of ethanol exposure inhibit dendritic development (Yanni and Lindsley, 2000; Lindsley et al., 2002) and delay initial axon outgrowth in these cultures, but that the rate of axon growth is increased overall due to shorter retractions that occur between bursts of elongation (Lindsley et al., 2003). We hypothesize that altered Rho GTPase activity may contribute to these effects of ethanol and consider our model system ideally suited to

investigating this idea. Neurons in these cultures express Rac1, Cdc42 and RhoA, and their intracellular distribution changes during development (Da Silva et al., 2004). In situ Rho GTPase binding assays can provide a sensitive assessment of Rho GTPase activation specifically within growth cones (Li et al., 2002; Woo and Gomez, 2006).

An important regulator of neuronal proliferation, morphogenesis, and survival is brain-derived neurotrophic factor (BDNF), and its potential role in FASD is the subject of considerable interest (reviewed by Davis, 2008). BDNF and its receptor, tyrosine kinase B (TrkB), are expressed by pyramidal neurons in the developing hippocampus in vivo and in vitro (Luikart et al., 2005; Swanwick et al., 2004). BDNF/TrkB signaling promotes axon elongation in developing hippocampal neurons (Ip et al., 1993; Labelle and Leclerc, 2000; Yoshimura et al., 2005), and TrkB signaling is inhibited by ethanol in a variety of neural cell systems (Hellmann et al., 2009; Li et al., 2004; Ohrtman et al., 2006), including slice cultures of neonatal rat hippocampus (Davis et al., 1999). However, the potential involvement of BDNF/TrkB signaling in disrupted axon growth resulting from exposure to ethanol has not been investigated. Recently, several lines of evidence point to the involvement of Rho GTPases in axonal and dendritic growth responses to BDNF (Cohen-Cory et al., 2010; Numakawa et al., 2010), including responses in hippocampal neurons (Zhou et al., 2008). Therefore, in addition to assessing ethanol effects on Rho GTPase activity in the absence of added neurotrophic factors, we also examined its effects on Rho GTPase activation stimulated by BDNF.

Materials and Methods

Neuronal Cultures and Ethanol Treatment

Hippocampal pyramidal neuron cultures were prepared from hippocampi of fetal Sprague-Dawley rats (Taconic Farms) at gestational day 19, as described by Kaech and Banker (2006). Briefly, hippocampi were dissected from the cerebral cortices and cleaned of meninges, then dissociated by treatment with trypsin (0.25%) and trituration with a fire-polished Pasteur pipette into a single cell suspension. Cells were plated at a density of 5650 cells/cm² in Minimal Essential Medium (MEM) with 10% heat-inactivated horse serum onto poly-L-lysine coated glass coverslips. After 3 hours the coverslips with neurons attached were transferred into dishes containing cortical astrocytes in which serum-free neuron maintenance medium (MEM with N2 supplements and 0.1 mM pyruvate; InVitrogen, Carlsbad, CA) was added 1-2 days before. Immediately before transferring the coverslips into the dishes with astrocytes, USP ethanol was added to the medium to achieve a final concentration of 11, 22, 43, or 87 mM. Controls received no ethanol. Control and ethanol-treated cultures were maintained for 1 day in modular incubator chambers at 36°C in 5.0% CO₂, saturated with water or water/ethanol at the target concentration in the medium. We previously showed this method maintains a nearly steady ethanol concentration in the medium for at least 7 days (Yanni et al., 2000). Cultures were used 24-26 hours after plating, when 70-80% of the neurons have formed a single axon (stage 3), identifiable by a total length greater than 40 µm and longer than the next longest neurite by at least 20 µm (Dotti et al., 1988). Some control and ethanol-exposed cultures were exposed to 50 ng/ml BDNF (Alamone Labs, Jerusalem, Israel) for 3 minutes just prior to fixation and staining, as this treatment was reported to maximally increase whole cell Rac1/Cdc42 activity in dissociated cortical neuron cultures (Yuan et al., 2003).

Immunocytochemistry

Coverslips from each treatment group were rinsed briefly in warm phosphate-buffered saline (PBS) and fixed for 12 minutes in warm 4.0% paraformaldehyde, 0.12 M sucrose in PBS, pH 7.4, then rinsed in PBS and permeabilized for 3 minutes in 0.15% Triton X-100 in PBS.

Neurons were incubated for 1 hour at room temperature with blocking solution (10% bovine serum albumin (BSA; SP-5050, Vector Laboratories, Burlingame, CA) in PBS to block nonspecific binding of reagents. To visualize the F-actin and microtubule cytoskeleton, neurons were incubated overnight at 4°C with a polyclonal rabbit anti-tubulin antibody (65095; ICN Biomedicals, Aurora, Ohio) diluted 1:100 in 3.0% BSA in PBS, rinsed and incubated for 1 hour at 36°C with Alexa Fluor 568 Phalloidin (A-12380; Molecular Probes, Eugene, OR) diluted 1:50 in 3.0% BSA in PBS.

For plasma membrane TrkB labeling, cultures were washed briefly with warm PBS, pH 7.4, then fixed for 1 minute at 37°C in 4.0% paraformaldehyde, 0.10% glutaraldehyde, 0.25 M sucrose in PBS, pH 7.4. Cells were blocked in 10% BSA in PBS for 1 hour at 37°C, then incubated with rabbit anti-TrkB (1:200; #07-225 Millipore, Temecula, CA) in 3.0% BSA in PBS overnight at 4°C. This antibody is specific for the extracellular domain of rat TrkB, and has been characterized by Western blotting and immunohistochemistry (Galvão et al., 2008). Cells were rinsed in PBS then incubated in goat anti-rabbit Alexa 488 (1:500; Molecular Probes, Eugene, OR) in 3.0% BSA in PBS for 1 h at 37°C. Coverslips were mounted in 50% glycerol, 50% PBS, 0.10% NaN₃, 2.5% 1,4-diazobicyclo-(2,2,2)-octane (to inhibit fading), pH 6.8 (aqueous mounting medium), and sealed with enamel. Omitting the primary antibody from the immunostaining protocols eliminated all detectable fluorescence.

In Situ Affinity Binding Assays to Detect Activated GTPases in Growth Cones

Labeling GTP-bound Rho GTPases in situ was performed essentially as described by Li et al., (2002). Briefly, after fixing for 20 mins and permeabilizing, neurons were rinsed in PBS and incubated overnight at 4°C with glutathione-S-transferase (GST) fused to the Rac1/Cdc42-binding domain of p21-activated protein kinase 1 (Pak1-PBD) (GST-Pak1-PBD; 5 µg/ml in 3.0% BSA in PBS; Pierce, Rockford, IL), or with GST fused to the Rhotekin-Rho binding domain (Rhotekin-RBD) (GST-Rhotekin-RBD; 5.0 µg/ml in 3.0% BSA in PBS; Pierce, Rockford, IL). Active GTP-bound Rac1 and Cdc42 bind specifically to Pak1-PBD and GTP-bound RhoA binds specifically to Rhotekin-RBD, thereby serving as activation-specific probes in this assay. After rinsing in PBS, neurons were next incubated overnight at 4°C with rabbit anti-GST (PC53; Calbiochem, La Jolla, CA) diluted 1:1000 in 3.0% BSA in PBS, then rinsed in PBS and incubated for 1 hour at 37°C with goat anti-rabbit Alexa 488 (A11008; Molecular Probes, Eugene, OR) diluted 1:500 in 3.0% BSA in PBS. After rinsing in PBS, and staining with Alexa Fluor 568 Phalloidin (described above), the coverslips were mounted on slides in aqueous mounting medium (see above) and sealed with enamel. Omitting the GST-fusion protein or the anti-GST primary antibody from the staining protocol eliminated all visible fluorescence emission in the 520-550 nm wavelength range.

Quantitative Morphometry and Fluorescence Analyses

Slides were blinded to the treatment prior to image capture and analyses to prevent bias. Digital images of axonal growth cone surface TrkB, or microtubules and F-actin, or F-actin and anti-GST fluorescence were captured using a 63×/1.25 numerical aperture oil objective lens on an Olympus (Tokyo, Japan) BX50 upright microscope equipped with an Optronics digital camera. To minimize the potential influence of non-biological factors on fluorescence intensity, the fixation and staining protocol, coverslip storage before and after staining, the duration of exposure to light and the image capture settings were the same for all treatment groups within an experiment.

Selection of neurons for image capture was conducted using a pre-determined sampling pattern covering the entire coverslip and morphological criteria for stage 3 pyramidal neurons, described by Dotti et al., (1988); an oval or pyramidal cell body with diameter of 15-20 µm, 2 or more processes 15-20 µm long, and one process ≥ 40 µm which identifies it

as the axon. Growth cones with obvious morphologic damage due to handling during staining, or that intermingled with neighboring processes, were not selected. The majority of neurons in each treatment group were undamaged and met these selection criteria. Images used for qualitative assessment were obtained with a Zeiss laser-scanning microscope 510 system and 63 $\times$ oil objective using the 488 and 568 nm excitations of the argon laser, 3.5 zoom and 3.0 μ m optical slices.

The area of the entire growth cone (delineated by F-actin staining) and the length of the axon (visualized by microtubule staining), were measured using the freehand selections tool in Image J after calibration with a stage micrometer. Fluorescence intensity of GST staining within a region of interest (ROI) in the central and transitional region of the growth cone, was measured using the mean gray value measurement tool in Image J software (NIH). Background fluorescence in an identical ROI, positioned outside the growth cone in the same field, was subtracted to obtain the mean fluorescence intensity per unit area in arbitrary numerical units.

Statistical Analyses

Each experiment was conducted at least 3 times, each time using a separate culture prepared from pooled hippocampi from the fetuses of a timed pregnant rat. Within each experiment, 30–45 neurons were analyzed per experimental condition. As similar results were obtained in separate experiments, the data were combined after recalculating each measurement as a percent of the mean control value for the experiment. For analyses of growth cone area measurements, statistical significance was assessed by the non-parametric Mann Whitney U Test, as the observed data did not follow a normal distribution when tested using the Kolmogorov-Smirnov test of normality ($p < 0.01$). Axon length and GST staining intensity data were normally distributed and were therefore analyzed using ANOVA to assess the main effect of ethanol and BDNF pretreatment, with Newman-Keuls posthoc comparisons. The relationship between growth cone area and GTPase activity was assessed by calculating the Spearman rank correlation coefficient. Statistical tests were performed using Statistica software (StatSoft, Tulsa, OK).

Results

Ethanol alters the size and morphology of axonal growth cones

Ethanol in the medium of cultured hippocampal neurons increases the rate of axon growth and alters axon growth dynamics (Lindsley et al., 2003), which presumably depends on reorganization of the microtubule and F-actin cytoskeleton that determines growth cone morphology as well as behavior. To directly assess whether ethanol alters the morphology of axonal growth cones, fetal rat hippocampal neurons maintained for 1 day in control medium or medium containing 11–87 mM ethanol were fixed and stained to visualize microtubules and F-actin and the overall size of the axonal growth cones was measured (Fig. 1). Microtubule staining can be used to trace the entire axon from the cell body to the central region of the growth cone, and F-actin staining provides good visualization of the morphologically complex peripheral region of the growth cone (Fig. 1A, B).

As expected, the mean axon length for neurons maintained in normal medium or medium containing 11 – 87 mM ethanol for 24 hrs after plating in the current study was comparable to axon length at 33 hrs after plating in our previous study (Lindsley et al., 2003), for which identical culture methods and ethanol exposure conditions were utilized. The mean length of axons in control cultures was 89 μ m ($\pm$ 5.7 SEM) and 95 μ m ($\pm$ 7.2 SEM) in cultures maintained in medium with 43 mM ethanol. These differences were not statistically significant ($p = 0.32$; $F = 1.16$; ANOVA).

The growth cones of neurons maintained in medium containing 22, 43 or 87 mM ethanol appeared to have more prominent lamellipodia and filopodia and indeed when growth cone surface area was measured were significantly larger, than neurons maintained in control medium (Fig. 1C and Fig. 2, F-Actin; Control vs Ethanol). These findings are consistent with an effect of ethanol that reorganizes the growth cone cytoskeleton, and implies that Rac1 and Cdc42 could be activated.

Distribution and relative levels of active Rho GTPases in axonal growth cones

Whole cell Rho GTPase activity has been shown to be sensitive to ethanol in a variety of cell types in vitro (Guash et al., 2003; Qian et al., 2003; Schaffert et al., 2006) and in vivo (Joshi et al., 2006). Although whole cell assays reveal the relative levels of active Rho GTPases in cultures or tissues, they cannot address whether ethanol alters Rho GTPase activity in growth cones, where these enzymes act on the F-actin cytoskeleton to regulate growth cone morphology and axon growth dynamics. Given that exposure to ethanol altered the morphology of growth cones, and our previous work showed altered extension and retraction dynamics in growing axons, we tested whether the same conditions of ethanol exposure also altered growth cone Rho GTPase activity. We performed in situ GTPase binding assays on neurons maintained for 1 day in control medium or medium containing ethanol. We also examined GTPase activation in control and ethanol exposed neurons stimulated by brief treatment with the TrkB ligand BDNF, as growth factors are well-known inducers of process growth mediated by Rho GTPases. Given that blood levels of alcohol in humans rarely reach 87 mM (400 mg/dL), these studies examined effects of ethanol in the culture medium at concentrations between 11-43 mM.

In control neurons, active GTP-bound Rac1/Cdc42 localized by GST-PBD was distributed throughout the distal axons and their growth cones (Fig. 2; Control). As expected, F-actin was concentrated in the peripheral domain of growth cones, where it was observed in spike-like filopodia extending from the crescent-shaped expanse of lamellipodia at the leading edge. The peripheral region of growth cones was delineated from the F-actin-poor central region by a narrow transitional zone with mesh-like F-actin networks and arcs. Within the growth cone, active Rac1/Cdc42 overlapped with F-actin-rich regions, especially in the transitional zone. Maintaining neurons in medium with 43 mM ethanol did not appear to affect either the overall distribution or amount of active Rac1/Cdc42 in growth cones, compared to control neurons (Fig. 2: Control vs. Ethanol). Whole cell and growth cone RhoA activity is thought to be low in neurons during process outgrowth (Govek et al., 2005). Consistent with this idea, GST-RBD in situ binding assay staining was less intense than GST-PDB staining in axonal growth cones of hippocampal neurons at this stage of development. Mean arbitrary units (AUs) for GST-PBD staining in growth cones of control cultures was 50 AUs, but was only 16 AUs for GST-RBD staining.

Previous reports have shown that brief application of BDNF increases whole cell Rac1 and Cdc42 activity in rat cerebellar granule cells (Jin et al. 2005) and in cortical neuron cultures (Yuan et al., 2003). We found that the application of BDNF (50 ng/ml for 3 minutes) to hippocampal neurons just prior to fixation appeared to increase active Rac1/Cdc42 in axonal growth cones, especially in the central and transitional zones (Fig. 2; Control vs. Control + BDNF). Interestingly, no similar increase in active Rac1/Cdc42 was observed in ethanol exposed neurons treated with BDNF (Fig. 2; Ethanol vs. Ethanol + BDNF).

Ethanol effects on growth cone area are not accompanied by changes in mean Rho GTPase activity

To quantitatively assay the effect of ethanol on activated Rac1/Cdc42 and on activated RhoA in growth cones, with and without BDNF treatment, we measured average GST-PBD

or GST-RBD fluorescence intensity in axonal growth cones from each treatment group. Ethanol, at concentrations of 11, 22 and 43 mM in the medium, had no effect on mean levels of active Rac1/Cdc42 or RhoA in the growth cones of neurons developing in these culture preparations (Fig. 3). Therefore, the dose-related increases in growth cone area observed in this study (Fig. 1), did not coincide with significant changes in growth cone Rho GTPase activity.

Ethanol alters growth cone Rho GTPase activation by BDNF

Next, we assessed the effect of ethanol on BDNF-stimulated Rac1/Cdc42 activation (Fig. 4A). As expected, BDNF treatment (50 ng/ml for 3 min) significantly increased the mean intensity of GST-PBD staining in the axonal growth cones of control cells, by 26% compared to control cells with no BDNF treatment. This indicates that BDNF activates Rac1/Cdc42 in the axonal growth cones of hippocampal pyramidal neurons when they are extending axons in vitro. Importantly, this BDNF-induced stimulation in Rac1/Cdc42 activation was inhibited by exposure to ethanol in the medium in a dose-dependent manner, with significantly less active Rac1/Cdc42 in the growth cones of neurons maintained in medium with 22 or 43 mM ethanol.

The Rho GTPases Rac1 and Cdc42 are thought to have antagonistic roles with RhoA GTPase during axon growth and guidance, the former promoting extension and the latter inducing retraction. So we assessed ethanol effects on active RhoA in axonal growth cones, with and without BDNF treatment (50 ng/ml for 3 min) (Fig. 4B). Treating control neurons with BDNF, which increased active Rac1/Cdc42 in growth cones, had no effect on active RhoA, which remained at low levels. However, BDNF treatment of neurons exposed to 43 mM ethanol, which completely prevented BDNF-stimulated Rac1/Cdc42 activation, significantly increased active RhoA in growth cones, by 24% compared to control neurons treated with BDNF. Thus, the reciprocal nature of Rac1/Cdc42 and RhoA activity appeared to be preserved in the growth cones of ethanol-treated neurons, even though Rho GTPase activation in response to BDNF was impaired.

To explore the strength of the association between growth cone area and active Rac1/Cdc42 under baseline and BDNF-stimulated conditions in our model system, we performed correlation coefficient analyses (Fig. 4C). The data, plotted as growth cone area versus GST-PBD fluorescence intensity for each growth cone in the sample population, showed a slight downward trend suggesting smaller growth cones have more activated Rac1/Cdc42, but the value of the correlation coefficient was low, with and without BDNF treatment ($r^2 = -0.038$ and -0.009 , respectively). Thus, there was no apparent linear correlation between surface area and amount of activated Rac1/Cdc42 in individual growth cones in hippocampal neurons under the culture conditions used in these studies.

Surface expression of TrkB receptors

Hippocampal neurons in dissociated cultures express full-length TrkB receptors, which are already distributed throughout the cell soma and along their developing processes 1 day after plating; in the soma, TrkB immunopositive staining is diffuse, but it is distinctly punctate along axonal processes (Swanwick et al., 2004). However, we considered the possibility that decreased Rac1/Cdc42 activation in response to BDNF might reflect the absence of TrkB on axonal growth cones on neurons maintained in medium with ethanol. To test this, we examined the expression of TrkB in axonal growth cones in our cultures, comparing control and ethanol exposed neurons, using a TrkB antibody specific for the extracellular domain of full-length TrkB (Fig. 5). In cultures maintained in control medium (0 mM) for 1 day, nearly all stage 3 pyramidal neurons expressed TrkB, which appeared as numerous discrete puncta along the axon shaft and growth cone. Exposure to 43 mM ethanol in the medium had no

detectable effect on this distribution. The size and number of puncta was not quantitatively assessed, but no notable differences were apparent by visual inspection. Additional quantification using more sensitive methods of detection (i.e. surface biotinylation assay) is needed to rule out more subtle, but potentially important differences in TrkB expression. Nevertheless, these results confirmed that TrkB was expressed on newly formed axons and their growth cones in these cultures, and showed that conditions of ethanol exposure that altered growth cone morphology did not substantially alter the amount or distribution of these receptors on the growth cone surface.

Discussion

In the present study, we exposed rat hippocampal pyramidal neuron cultures to 11, 22, 43 mM, and in one experiment 87 mM, ethanol in the medium for 1 day during early axonogenesis. This range of ethanol concentration was roughly equivalent to low-moderate to high blood alcohol concentrations (50 to 400 mg/dl) that are commensurate with levels reported in chronic alcoholics and correlated with risk of fetal CNS damage (Maier and West, 2001). These conditions replicated those we previously found increased the overall rate of axon elongation, without affecting neuronal survival (Lindsley et al., 2003). The current studies examined the effects of ethanol on axon growth cone morphology and on levels of active Rho GTPases in these cultures, with and without activation of Rac1/Cdc42 GTPases by BDNF.

Ethanol and Growth Cone Rho GTPase Activity: Without Added BDNF

Previous studies have established that ethanol can alter Rho GTPase activity in whole cell lysates of non-neuronal and neuronal cell types, but to our knowledge this is the first study to measure levels of Rho GTPases within axonal growth cones. Here, we report that ethanol increased the surface area of growth cones in a dose-dependent manner, which is consistent with activation of Rac1 and Cdc42 that has been shown in other studies to correlate with protrusion of F-actin-rich lamellipodia and filopodia in the growth cone. Thus, it was surprising when we found that levels of active Rho GTPases in axonal growth cones were not affected by ethanol at any dose tested. The absence of detectable changes in Rac1/Cdc42 or RhoA GTPase activity in this study suggest that the altered growth dynamics previously reported under these same conditions may not coincide with global alterations in Rho GTPase activity in growth cones. However, our analyses might not detect an effect limited to a particular phase of saltatory axon growth, as the analyses of a population of fixed axons prevented us from knowing whether a given growth cone was growing, pausing or retracting at the time of fixation. Although fluorescence resonance energy transfer (FRET) analyses of Rho GTPase activation in living cells has been reported (Aoki et al., 2004), methods are not yet available for long-term time-lapse observation of growth dynamics in neurons expressing Rho GTPases in their native distribution in the cytoplasm.

Ethanol and Growth Cone Rho GTPase Activity: Response to BDNF

In contrast to results obtained when the conditions of ethanol exposure mimicked those of our previous work, we found that ethanol exposure *did* alter growth cone Rho GTPase activation induced by BDNF; inhibiting Rac1/Cdc42 activation in a dose-dependent manner, and increasing RhoA activation at the highest concentration tested. This is the first study to assess the effects of ethanol on BDNF/TrkB signaling in this culture model for which ethanol effects on axonal and dendritic development are well described. Overall, our findings are consistent with studies that have examined effects of ethanol on BDNF/TrkB signaling in other preparations, the majority of which found inhibitory effects (reviewed in Davis et al., 2009). Despite considerable interest in the possible role of BDNF/TrkB in FASD, the mechanism underlying these effects is poorly understood and most studies have

focused on signaling that mediates BDNF effects on cell proliferation and survival, not morphologic effects.

Our observation that ethanol inhibits BDNF-induced Rac1/Cdc42 is in line with the findings reported by Joshi and colleagues (2006), who showed that whole cell lysates of cerebellar tissue from P7 rats exposed to ethanol resulting in blood levels of 40 mM or 80 mM, had less activated GTP-bound Rac1 and, at the higher alcohol concentration, also had more activated RhoA than lysates from control rat cerebella; a relevant comparison given that tissue in vivo would be stimulated by endogenous growth factors, including neurotrophins. On the other hand, our failure to detect Rho GTPase activity in growth cones without BDNF stimulation differs from the inhibition of Rac1 and activation of RhoA they observed when granule cells from ethanol-exposed pups were examined in vitro. These differences could point to effects of ethanol on Rho GTPase activity in tissue or whole cell lysates that are not reflected in axonal growth cones, however, they could instead be due to different cell types or culture conditions. Since the effects of ethanol on process outgrowth in vitro are stimulatory for hippocampal neurons (Lindsley et al., 2003) and inhibitory for cerebellar granule cells, it is reasonable to expect that disparate effects on Rho GTPase activity that regulate neuritogenesis might reflect these differences. Although processes grow on polylysine-coated substrates in both model systems, the depolarizing conditions used to promote neurite outgrowth of cerebellar neurons may have activated Rho GTPases, as pointed out by the authors (Joshi et al., 2006). Considering evidence that tightly balanced and localized levels of Rac1 and RhoA activity promote neurite elongation at optimal levels, and increasing or decreasing activity is inhibitory to growth, it is tempting to speculate that the opposing effects of ethanol in these systems may be attributable in part to different levels of active Rac1.

In our model system rat cortical astrocytes provide diffusible factors in the culture medium that support neuronal survival and differentiation (Kaech and Banker, 2006). The astrocytes are present as a monolayer in the dishes, but are physically separated from the neurons growing on coverslips. We previously reported that inhibitory effects of ethanol on dendritic development of neurons in this model system are independent of astrocyte responses to ethanol (Yanni and Lindsley, 2002), but other neuronal responses to ethanol may be mediated indirectly by astrocytes. In the present study we observed no effect of ethanol on Rho GTPase activity in the absence of added BDNF, but we can not rule out the possibility that altered responsiveness to BDNF could nevertheless be indirectly related to effects on the neurons that were mediated by the astrocytes.

Some studies have reported developmental ethanol exposure can transiently reduce TrkB expression in hippocampus (Moore et al., 2004), whereas others reported no changes (Feng et al., 2005). We think the mechanism underlying ethanol inhibition of Rac1/Cdc42 activation by BDNF in the current study is not likely to involve large changes in surface TrkB receptor expression, as we observed no obvious differences in the distribution or amount of TrkB using an antibody recognizing an extracellular domain of the receptor. However, more quantitative methods might detect small, but potentially important, changes in expression or distribution of TrkB on growth cones.

Rac1/Cdc42 Activity and Growth Cone Area

It is noteworthy that Rac1/Cdc42 activity and growth cone area were not correlated in these studies. The apparent disconnect between surface area and Rac1/Cdc42 activation is surprising, given many reports that growth cone expansion is correlated with activation of Rac1 and Cdc42 (Kozma et al., 1997; Shekarabi and Kennedy, 2002), and that active Rac1 and Cdc42 promote axon extension (reviewed in Govek et al., 2005). Indeed, the association between Rac1/Cdc42 activation and membrane expansion appears to be true in hepatocyte

cultures which display cell spreading profiles that closely correlate with whole cell Rac/Cdc42 activation, regardless of whether they are prepared from control-fed or ethanol fed rats (Schaffert et al., 2006).

On the other hand, the same GTPases can have opposite effects on growth cone behavior. For example, RhoA activation results in growth cone collapse and axon retraction in hippocampal neurons on a laminin substrate (Da Silva et al., 2003), but enhances growth cone protrusions and axon elongation in *Xenopus* retinal neurons growing on laminin (Woo and Gomez, 2006). In addition, growth cone expansion and axon outgrowth are not always correlated in response to acute treatment with BDNF that activates Rac1/Cdc42. For example, *Xenopus* spinal neurons in culture for 6 hrs collapse and retract in response to BDNF, but 24 hr cultures grow. Both responses are modulated by cAMP; inhibition of cAMP leads to collapse and increasing cAMP blocks collapse (Wang and Zheng, 1998). Thus, whether response to BDNF enhances or inhibits extension may depend on intracellular cAMP levels. Given that ethanol can modulate cAMP signaling (Tabakoff et al., 2001), the possibility that ethanol exposure alters BDNF signaling involves altered cAMP levels in growth cones deserves further study.

Possible Mechanisms Underlying Ethanol Effects on Rho GTPase Response to BDNF

If TrkB expression and Rho GTPase activity in the absence of BDNF stimulation is not significantly altered by ethanol, then what could account for reduced Rac1/Cdc42 GTPase response to BDNF? In addition to cAMP, mentioned above, calcium reportedly modulates TrkB signaling without affecting receptor function per se. Regulation of Cdc42 activity by BDNF in cerebellar neurons requires elevation of intracellular calcium (Jin et al., 2005). Indeed, in developing rat CA3 hippocampal pyramidal neurons, ethanol inhibits BDNF-dependent LTP-GABA_A via inhibition of L-type calcium channels (Zucca and Valenzuela, 2010) and depolarization-induced calcium flux in axonal growth cones is inhibited by ethanol (our unpublished observations). Effects of neurotrophins on cell motility are also dependent on the recruitment of downstream signaling molecules to plasma membrane microdomains called lipid rafts. For example, in cerebellar granule cell cultures neurite outgrowth mediated by the L1 cell adhesion molecule is inhibited by ethanol (Bearer et al., 1999), and L1 signaling induced by clustering in the plasma membrane through MAP kinases requires Rac1-mediated Pak1 activation (Schmid et al., 2004). Similarly, in hippocampal neurons and PC12 cells, NGF-induced F-actin reorganization and activation of Rac1 is inhibited by preventing Rac1 recruitment to lipid rafts (Fujitani et al., 2005). Ethanol disrupts lipid rafts in a variety of cell types (Szabo et al., 2007), including cerebellar granule neurons (C. Bearer, personal communication), raising the possibility that a similar mechanism could contribute to impaired BDNF/TrkB activation of Rac1/Cdc42 in the growth cones of ethanol-exposed hippocampal neurons.

Implications of the Current Findings

Our current findings add to growing evidence supporting a role for Rho GTPases in ethanol-induced actin remodeling and changes in cell shape and motility. For example, actin reorganization and changes in cell shape appear to be mediated by RhoA in cortical astrocyte cultures treated with ethanol, as these effects are prevented by expression of constitutively active RhoA (Guash et al., 2003). Chronic ethanol feeding impairs Rac1 and Cdc42 activation and cell–extracellular matrix adhesion events in rat hepatocytes, but has no effects on RhoA (Schaffert et al., 2006). Activation of Cdc42 in SVEC4-10 endothelial cell cultures mediates actin filament reorganization and altered cell motility induced by acute ethanol exposure, and these effects are prevented by dominant negative Cdc42 (Qian et al., 2003).

Our finding that ethanol impairs BDNF/TrkB signaling in growth cones has broader implications than axonal growth. The impaired growth cone response to BDNF in axons of hippocampal pyramidal neurons exposed to ethanol has implications for disruption of their pathfinding as well, as BDNF is a chemoattractant for these axons (Mai et al., 2009). BDNF also has a role in mossy fiber pathfinding from the dentate gyrus to the CA3 region, as shown by experiments in which pharmacologic inhibition of Trk signaling in hippocampal slice cultures induces hypertrophic extension of mossy fibers (Tamura et al., 2009). This raises the possibility that impaired BDNF/TrkB signaling may contribute to the aberrant mossy fiber trajectories observed in the hippocampus of rats exposed to ethanol during development (West et al., 1981; West and Hamre KM, 1995; Sakata-Haga et al., 2003).

Further elucidation of the mechanisms by which Rho GTPases contribute to ethanol effects on signaling that regulates axon growth and guidance will improve our understanding of how ethanol disrupts neural development. This may reveal potential targets for new therapeutic strategies to prevent prenatal damage in the fetus of pregnant alcoholics who are unable to abstain from alcohol, and to reverse these effects in alcohol-affected children. Evidence supporting the therapeutic use of BDNF for reversing abnormal process outgrowth in neurons developing in vitro after prenatal hypoxia (Briscoe et al., 2006) suggests BDNF signaling might also be a therapeutic target for process growth and guidance defects associated with FASD.

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Fig. 1.

Ethanol increases the surface area of axonal growth cones in rat hippocampal neuron cultures. (A) A representative stage 3 neuron immunostained to visualize microtubules (green) and F-actin (red), and measured to confirm that it has a single axon at least 40 μm long. (B) A higher magnification image of the axonal growth cone in A and tracing delineating the boundaries of the ROI used to measure its surface area. (C) Quantification of growth cone area in stage 3 neurons. Exposure to 11, 22, 43 or 87 mM ethanol in the medium for 1 day beginning at plating increased the surface area of axonal growth cones in a dose-dependent manner. Data are the mean area and SEM from the combined results of 6 separate cell preparations (a total of 576 neurons measured), and are expressed as percent of 0 mM ethanol (% Control); * $p < 0.05$ and ** $p < 0.005$ compared to 0 mM; Mann Whitney U Test. Scale bar = 10 μm

Fig. 2.

In situ activation assays of activated Rac1/Cdc42 Rho GTPases in axonal growth cones. Shown are representative confocal microscope images of axonal growth cones stained to visualize F-actin (red) and GST-PBD (green), which binds to GTP-bound Rac1 and Cdc42 and is detected using anti-GST antibodies. Ethanol (43 mM) appeared to increase lamellipodia and filopodia in growth cones compared to controls, but did not appear to affect amount or distribution of GTP-bound Rac1/Cdc42 in growth cones. An increase in apparent intensity of GST-PBD staining induced by treatment with BDNF (50 ng/ml for 3 min) was observed in control cells, but not in cells maintained in medium containing 43 mM ethanol. Scale bar = 5 μ m.

Fig. 3.

Quantification of active Rac1/Cdc42 and active RhoA in axonal growth cones, as indicated by intensity of GST-PBD and GST-RBD staining, respectively. Exposure to ethanol for 1 day beginning at plating had no effect on levels of active Rac1/Cdc42 or RhoA in an ROI in the central and transitional zones of axonal growth cones. Plotted is the mean (+ SEM) arbitrary fluorescence intensity per unit area within the growth cone after background subtraction. Data are the combined results of 3 separate cultures ($n = 56-65$ growth cones per treatment group), expressed as percent of 0 mM ethanol and analyzed by ANOVA with Newman-Keuls post hoc tests.

Fig. 4.

Exposure to ethanol for 1 day beginning at plating alters Rac1/Cdc42 and RhoA GTPase response to BDNF within axonal growth cones. (A) Quantification of Rac1 and Cdc42 activation in axonal growth cones, as indicated by intensity of GST-PBD staining per unit area. BDNF (50 ng/ml for 3 min) increased Rac1/Cdc42 activity in the growth cones of control neurons (* $p < 0.005$; ANOVA with Newman-Keuls posthoc, compared to 0 mM Ethanol and no BDNF). Ethanol exposure inhibited BDNF-induced activation of Rac1/Cdc42 in a dose-dependent manner (# $p < 0.05$; ANOVA with Newman-Keuls posthoc, compared to 0 mM Ethanol and BDNF treatment). Data are the mean (+ SEM) from the combined results of 3 separate cultures, expressed as percent of 0 mM ethanol and no BDNF

treatment. (B) Quantification of RhoA activation in axonal growth cones, as indicated by intensity of GST- RBD staining in the growth cone. Exposure to 43 mM ethanol increased activation of RhoA within axonal growth cones. (* $p < 0.05$; ANOVA with Newman-Keuls posthoc, compared to 0 mM Ethanol and BDNF treatment). Plotted is the mean (+ SEM) intensity per unit area in the growth cone. Data are the combined results of 3 separate cultures, expressed as percent of 0 mM ethanol with no BDNF treatment. (C) The correlation coefficient of determination (r^2) between growth cone area and intensity of GST-PBD staining, indicates no relationship between growth area and Rac1/Cdc42 activity in neurons, regardless of BDNF stimulation. Data are the raw values for data plotted in A.

Fig. 5. TrkB expression in axonal growth cones of stage 3 hippocampal neurons maintained in control medium or medium containing ethanol. TrkB protein was detected using an antibody specific for the extracellular domain of TrkB (Millipore cat. # 07-225). Staining was detected in the distal axon and growth cone of control and ethanol-treated neurons, without notable differences in amount or distribution. Scale bar = 10 μ m