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Research Article

Ethanol impairs Rho GTPase signaling and differentiation of cerebellar granule neurons in a rodent model of fetal alcohol syndrome

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Abstract. Developmental exposure to ethanol impairs fetal brain development and causes fetal alcohol syndrome. Although the cerebellum is one of the most alcohol-sensitive brain areas, signaling mechanisms underlying the deleterious effects of ethanol on developing cerebellar granule neurons (CGNs) are largely unknown. Here we describe the effects of *in vivo* ethanol exposure on neurite formation in CGNs and on the activation of Rho GTPases (RhoA and Rac1), regulators of neurite formation. Exposure of 7-day-old rat pups to ethanol for 3 h moderately increased blood alcohol concentration (BAC) (~40 mM) and inhibited neurite formation and Rac1 activation in CGNs. Longer exposure to ethanol for 5 h resulted in higher BAC (~80 mM), induced apoptosis, inhibited Rac1, and activated RhoA. Studies demonstrated a regulatory role of Rho GTPases in differentiation of cerebellar neurons, and indicated that ethanol-associated impairment of Rho GTPase signaling might contribute to brain defects observed in fetal alcohol syndrome.

Keywords. Cerebellar granular neurons, RhoA, Rac1, fetal alcohol syndrome, neuronal differentiation.

Introduction

Fetal alcohol syndrome (FAS) is a human disorder characterized by dysmorphogenesis, central nervous system (CNS) disturbances, and craniofacial abnormalities, and results from intrauterine exposure to ethanol. Children exposed prenatally to ethanol often manifest anatomical features linked to FAS, including microcephaly; microencephaly; CNS disorganization; corpus callosum, basal ganglia, and cerebellum anomalies; and neuroglial heterotopias [1–3]. Children with FAS also show delayed motor development, problems performing fine motor tasks, and ataxia, indicative of cerebellar damage [4, 5]. Indeed, MRI studies have revealed significant reductions in cerebellar volume in individuals with FAS [6, 7].

Although the legal intoxication limit in the United States is 80–100 mg/dL (~20 mM), blood alcohol concentrations (BAC) as high as 500 mg/dL (~110 mM) have been reported in intoxicated individuals [8–10]. Approximately 15% of women of child-bearing age who consume alcohol are moderate to heavy drinkers, and about 13% of these women continue to use alcohol during pregnancy [11, 12]. Since ethanol readily crosses the placental and blood-brain barrier, diffusing rapidly into all aqueous

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compartments within the body [13], fetuses of women who abuse alcohol would readily be exposed to moderate-to-high alcohol concentrations.

Neurons are particularly sensitive to ethanol-related toxicity during the synaptogenesis phase of brain development (also referred to as the 'brain growth spurt' stage), which is characterized by neurite elaboration, synapse formation, and onset of neuron-neuron signaling [14]. In humans, synaptogenesis begins during the third trimester of pregnancy and continues through the first few years of life [15]. In rodents, this period corresponds to postnatal days 4–9 (P4–P9); a single ethanol exposure during this period depletes neurons [16]. The third trimester is also important with respect to cerebellar development, and exposure of rat pups to ethanol during the third-trimester equivalent has been shown to reduce cerebellar size [17, 18].

Although ethanol toxicity affects both cerebellar Purkinje cells and granule neurons [16, 19], susceptibility to ethanol-associated damage peaks during P4–P6 in Purkinje cells [20]. Cerebellar granule neurons (CGNs), on the other hand, remain susceptible until they migrate from the external to the internal granule layer, which occurs between P7 and P12 [21–23]. Alcohol reduces survival and impairs migration and axon guidance of CGNs [19, 23–26]. Neurite network formation that ultimately results in synaptic connections is important in all neurons including the CGNs, since their survival is activity dependent [27]. Studies using cultured CGNs exposed to ethanol have shown that immature neurons are more susceptible to ethanol toxicity than are mature neurons [28], indicating that alcohol may also affect differentiation. It has been reported recently that exposure of CGNs to moderate ethanol concentration does not induce apoptosis, but does inhibit neurite formation as well as secondary branching [29].

Small molecular weight G-proteins of the Rho family (RhoA, Rac1, and Cdc42) regulate neurite formation [30]. Rac1 and Cdc42 positively affect neurite extension, whereas RhoA mediates neurite retraction [31–33]. These proteins regulate the cytoskeletal rearrangement (formation of stress fibers and focal adhesion complexes) necessary to achieve neurite formation [34]. Rho GTPases also regulate neuronal polarization and plasticity important for cognitive functions [30]. Ethanol affects cognitive functions and the precise connectivity among neurons necessary for normal brain function [35, 36]. These observations led us to hypothesize that altered Rho GTPase signaling may underlie the toxic effects of ethanol, resulting in impaired neurite formation.

In the present study, we used a rodent model of FAS to examine the effects of ethanol on neurite formation in CGNs. Rat pups (P7) were exposed to either moderate or high concentrations of ethanol, concentrations typically found in pregnant women who drink moderately to heavily. We subsequently examined neurite outgrowth formation and Rac1 and RhoA activation in CGNs isolated from the ethanol-exposed pups. To confirm the role of Rac1 and RhoA in neurite outgrowth, we assessed neurite formation in cells constitutively overexpressing active and dominant negative forms of Rac1 and RhoA. Here we report that ethanol exposure alters Rho GTPase signaling and inhibits neurite formation, indicating that some of the toxic effects of ethanol result from impaired Rho GTPase signaling.

Materials and methods

Preparation of primary CGN cultures. All animals used in these studies were handled in accordance with national guidelines for animal welfare, which are consistent with the ethical principles and guidelines for scientific experiments on animals laid down by Swiss Academy of Medical Sciences. The present study was performed using 7-day-old (P7) Sprague Dawley rat pups obtained from five different litters. An average of 14 pups was obtained from each litter.

Primary CGN cultures were prepared from P7 Sprague Dawley rats as described previously [19]. Briefly, cerebellar tissue was dissected out from 12 pups/litter (6 control and 6 ethanol exposed pups), enzymatically dissociated with trypsin at 37 °C for 15 min, then treated with soybean trypsin inhibitor (Worthington, Lakewood, NJ) and DNase I (Worthington, Lakewood, NJ) for 5 min. Cells were dissociated by trituration, washed once with Basal Medium Eagles (BME) containing 25 mM KCl, and plated at a density of 0.5×10^6 cells/cm^2 in BME containing 10% fetal bovine serum (FBS). Tissue culture plates coated with poly-L-lysine (10 µg/mL) were used. For immunochemical studies, neurons were plated on glass chamber slides coated with poly-L-lysine (10 µg/mL)/laminin (1 µg/mL). After 12–14 h, 10 µM cytosine-D-arabinofuranoside (Sigma, St. Louis, MO) was added to the cultures to prevent growth of non-neuronal cells. With this protocol, nearly 95% of the cells counted in each batch were CGNs. Neuronal cells are phase bright, whereas non-neuronal cells are firmly adherent and dark.

Exposure of neonatal rat pups to ethanol. P7 rat pups were exposed to alcohol through inhalation of ethanol-saturated air as described previously [37]. This method of administering alcohol produces CNS anomalies comparable to those produced by other routes of administration [38, 39]. Pups (6 pups/litter) along with nursing dams were placed in an inhalation chamber for either 3 or 5 h. For controls, similar numbers of pups were placed in another chamber without exposing them to ethanol. Pups were given milk formula (0.2 mL/pup) every 2 h through-

out the alcohol exposure period. The hands were slowly moved into the inhalation chamber through the hand slits present in the inhalation chamber. The pups were hand fed with the help of a catheter tapered like a nipple. The catheter was lubricated outside with corn oil to prevent any damage to the esophagus [40]. This procedure allowed us to feed the pups while they are inside the inhalation chamber and also it did not required any administration of anesthesia. At the end of the inhalation period, we estimated BAC with an alcohol reagent kit (Pointe Scientific, Canton, MI) according to the manufacturer's instructions. Mean BAC values were calculated from BACs obtained from three sets of pups (6 pups/set). Exposure of pups to ethanol for 3 h resulted in moderate blood alcohol levels (183 ± 3.78 mg/dL or ~40 mM), while exposure of pups to ethanol for 5 h resulted in higher blood alcohol levels (384.75 ± 52.6 mg/dL or ~80 mM).

For immunohistological studies, cerebellar tissue was harvested at the end of ethanol exposure and fixed in 10% formalin for 24 h.

To study the effect of ethanol exposure to CGNs, cerebellar tissue from all 6 pups was harvested together and CGNs were isolated. CGNs were subsequently cultured for 2 h in low serum (3%) to keep the basal activation of Rho GTPases low. At the end of the 2-h incubation, CGNs were used to study activation of RhoA and Rac1 and for immunochemical studies. To rule out ethanol-withdrawal effects [41] on Rac1 and RhoA activation, we included either moderate (183 mg/dL) or high (384.75 mg/dL) concentrations of ethanol into the media throughout the CGN isolation and culturing procedure. To monitor neurite outgrowth, CGNs from ethanol-exposed pups were cultured in a medium containing low serum and ethanol (183 mg/dL) for 24 h. The cultures were placed in sealed containers with equimolar ethanol in another petri dish to prevent loss of ethanol due to evaporation [42].

Histochemical analysis. Cerebellar tissue was dissected out, fixed in 10% formalin for 24 h, embedded in paraffin, and sectioned with a microtome (thickness 6 μm). Sections were re-hydrated with graded ethanols. To detect the presence of apoptotic nuclei, sections were stained using a TUNEL labeling kit (Promega, Madison, WI) according to the manufacturer's protocol. Sections were also stained for actin by using Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR). (data not shown).

Cell lysis and Western blot analysis. Total cellular proteins were isolated using cell lysis buffer (Cell Signaling Technology, Beverly, MA) and Western blotted as described previously [43, 44].

Assay of RhoA and Rac1 activation. We analyzed RhoA and Rac1 activation using pull-down assays of active forms of RhoA and Rac1. Glutathione-S-transferase fused Rhotekin binding domain (GST-RBD) and GST-PAK binding domain (GST-PBD) were used to pull-down GTP-RhoA and GTP-Rac1, respectively, from the cell lysates [43, 44]. Briefly, cell lysates were incubated with either GST-RBD or GST-PBD immobilized on agarose beads for 1 h, washed three times with lysis buffer, and Western blotted for either RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) or Rac1 (Cell Signaling Technology, Beverly, MA) respectively, to detect the GTP-bound form of these proteins. Total RhoA and Rac1 levels in corresponding cell lysates were also determined by Western blotting for normalization of GTP-bound Rac1 and GTP-bound RhoA levels. Intensity of signals corresponding to GTP-Rac1 in GST-PBD assay and total Rac1 in the cell lysates was determined by scanning densitometry. The ratio of GTP-Rac1 and total Rac1 signal intensity was the amount of active Rac1. Increase or decrease in the amount of active Rac1 after ethanol administration was expressed relative to the amount of active Rac1 in ethanol-untreated control samples. Activation/inactivation of RhoA after ethanol administration was calculated similarly. The assay was performed in duplicates and the experiment was repeated in five different litters.

Indirect immunofluorescence microscopy. Focal adhesions were detected by immunostaining cultured CGNs with anti-vinculin antibody (1 : 200; Sigma) followed by FITC-conjugated goat anti-mouse IgG (1 : 100; Santa Cruz Biotechnology). Polymerized actin was detected with Texas Red-conjugated phalloidin (1 : 200) as described previously [44]. Staining was examined with an Olympus Provis microscope (60× magnification) using epifluorescence illumination.

Adenoviral infection. CGNs (1 day *in vitro*) were infected with adenovirus expressing green fluorescent protein (GFP)-fused Rac1 V12, GFP-Rac1 N17, GFP-RhoA V14, or GTP-RhoA N19 at multiplicity of infection (moi) of 100 for 48 h. Cells infected with adenovirus expressing GFP were used as controls. This protocol resulted in a 95% infection rate, as indicated by the number of GFP-expressing cells *versus* non-GFP-expressing cells (data not shown).

MTT assay. We assessed the viability of CGNs isolated from ethanol-exposed pups using an MTT assay kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Neurons isolated from ethanol-exposed pups were incubated with MTT reagent for 4 h and then incubated overnight in solubilization buffer. Absorbance of the formazan product was read at 575 nm. A reference wavelength of 650 nm was used to detect background. In this assay the amount of formazan formed due to cell metabolism is a direct measure of cell viability [45].

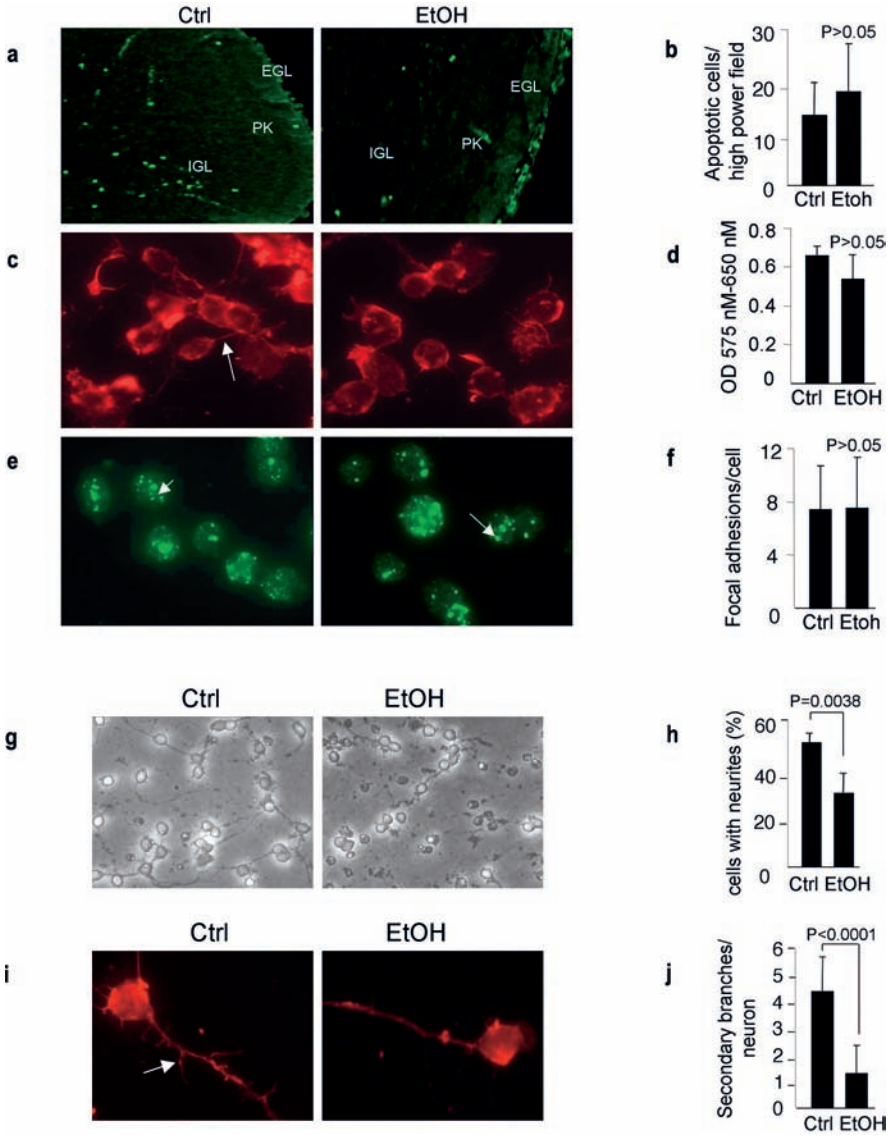

Figure 1. Exposure to moderate ethanol concentrations stunts neurite formation in cerebellar granule neurons (CGNs). Rat pups at postnatal day 7 (P7) were exposed to ethanol for 3 h, and CGNs were either isolated for cell culture or cerebellar tissue was fixed for histology. (*a*) Tissue sections from unexposed and ethanol-exposed pups showing TUNEL-stained apoptotic cells. The number of TUNEL-stained cerebellar cells was comparable in tissue unexposed (control) and exposed to ethanol. The external granule layer (EGL), Purkinje cell layer, and internal granule layer (IGL) are marked. (*b*) Number of apoptotic cells was counted from five random fields/brain sections and an average from three pups belonging to five different litters was plotted. (*c*) Actin-stained CGNs cultured *in vitro* for 2 h. Initiation of small neurites (indicated by arrows) was inhibited in CGNs derived from ethanol-exposed pups. (*d*) MTT assay of CGNs from ethanol-exposed pups revealed that cell survival was unaffected by moderate ethanol concentrations. The assay was performed with 12 replicates and the experiment was repeated in five separate litters. Values represent mean ± SD. (*e*) Focal adhesion complexes (indicated by arrow) were visualized with mouse anti-vinculin IgG and FITC-conjugated anti-mouse IgG. The formation of focal adhesions was not affected by moderate ethanol exposure. (*f*) Number of focal adhesions per cells was counted from five fields each and an average from five different litters was plotted. (*g*) Phase-contrast photomicrographs of CGNs isolated from ethanol-exposed pups (20× magnification). The CGNs were incubated in medium containing 183 ± 3.78 mg/dL or ~40 mM ethanol for 24 h. (*h*) The percentage of cells bearing neurites was counted from five random fields/plate and an average from five different litters was calculated. Ethanol exposure inhibits neurite formation. Exposure of pups to ethanol resulted in fewer CGNs that developed neurites. (*i*) Actin-stained CGNs from ethanol-exposed pups cultured *in vitro* for 24 h. Ethanol exposure inhibits secondary neurite branching. (*j*) Neurites of CGNs from ethanol-exposed pups has fewer secondary branches compared with those of CGNs from unexposed pups (control). The number of secondary branches was counted in ten lone neurons in triplicate per litter and an average from five different litters was plotted.

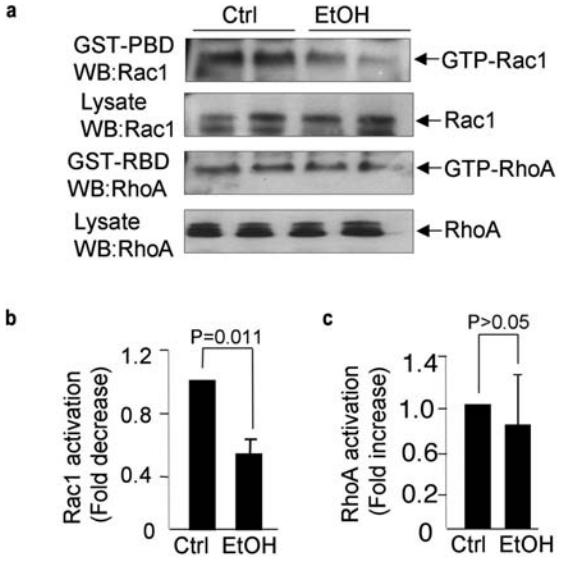

Figure 2. Exposure to moderate ethanol concentrations inhibits Rac1 in CGNs. P7 rat pups were exposed to ethanol-saturated air for 3 h. CGNs were isolated and cultured *in vitro* or 2 h. (*a*) Western blots of affinity-precipitated (GST-PBD and GST-RBD) CGN lysates from ethanol-exposed rats. Rac1 activation was inhibited, while RhoA activation was unaltered. Active forms of Rac1 and RhoA were precipitated using GST-PBD- and GST-RBD-conjugated beads, respectively, and Western blotted for Rac1 and RhoA (GST-RBD, glutathione-S-transferase-fused Rhotekin binding domain; GST-PBD, GST-PAK binding domain). (*b*) Ethanol-induced inhibition of Rac1 in CGNs. Bands corresponding to Rac1 was estimated using scanning densitometry. Activation of Rac1 was calculated as the ratio of GTP-Rac1 to total Rac1 and expressed as fold increase/decrease with respect to Rac1 activation in CGNs isolated from control pups normalized to onefold. (*c*) Ethanol-induced activation of RhoA in CGNs. Activation of RhoA was similarly calculated by densitometric scanning of signals corresponding to GTP-RhoA and RhoA. The values represent mean ± SD from five different litters.

Statistical analysis. Statistical significance of the data was determined by one-way analysis of variance (ANOVA) and paired *t*-tests. The results were considered significant at $p < 0.05$.

Results

Ethanol inhibits neurite formation in CGNs. Rat pups (P7) exposed to ethanol for 3 h had moderate BACs (183 ± 3.78 mg/dL or ~40 mM). Cerebellar tissue sections from ethanol-exposed and unexposed pups were TUNEL stained to detect the presence of apoptotic nuclei. The number of TUNEL-positive cells in the external granule layer, Purkinje cell layer, and internal granule layer was comparable to that in unexposed control cerebellar tissue, indicating that moderate ethanol concentrations did not induce apoptosis (Fig. 1a, b). Initiation of neurite processes was studied in CGNs from ethanol-exposed or unexposed pups by staining for actin, a component of the

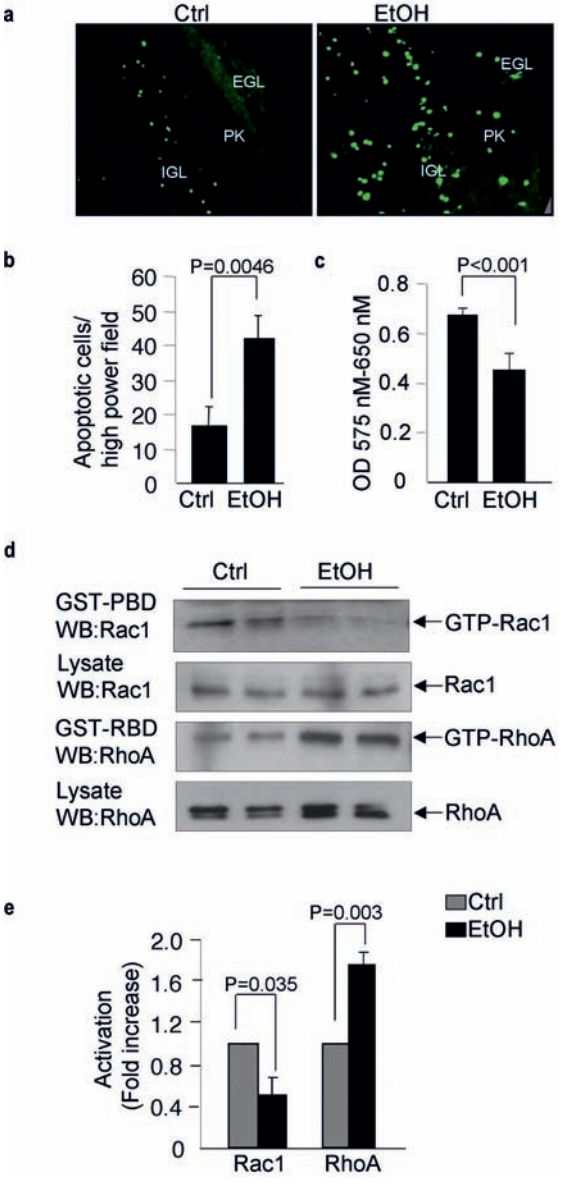

Figure 3. CGNs from rat pups exposed to high ethanol concentrations show inhibition of Rac1 and activation of RhoA. P7 rat pups were exposed to ethanol for 5 h after which cerebellar tissue was fixed or CGNs isolated and cultured *in vitro* for 2 h. (*a*) TUNEL-stained sections from ethanol-exposed pups contained more apoptotic cells than did sections from unexposed pups. The external granule layer (EGL), Purkinje cell layer and internal granule layer (IGL) are indicated. (*b*) Histogram showing number of apoptotic cells in TUNEL-stained cerebellar sections from ethanol exposed pups. The number of apoptotic cells was counted in eight random fields/brain section and an average from five pups (one from each litter) was counted. (*c*) MTT assay of CGNs isolated from ethanol-exposed pups indicated reduced CGN survival. The assay was performed with 12 replicates and the experiment was repeated in five separate litters. Values represent mean ± SD. (*d*) Rac1 was inhibited, while RhoA was activated in CGNs from ethanol-exposed pups. Active forms of RhoA and Rac1 were precipitated using GST-RBD and GST-PBD respectively, as described in Materials and Methods, and Western blotted. (*e*) Activation of RhoA and Rac1 was calculated by densitometric scanning of corresponding bands as described in the legend of Fig. 2b. The values represent mean ± SD from five different litters.

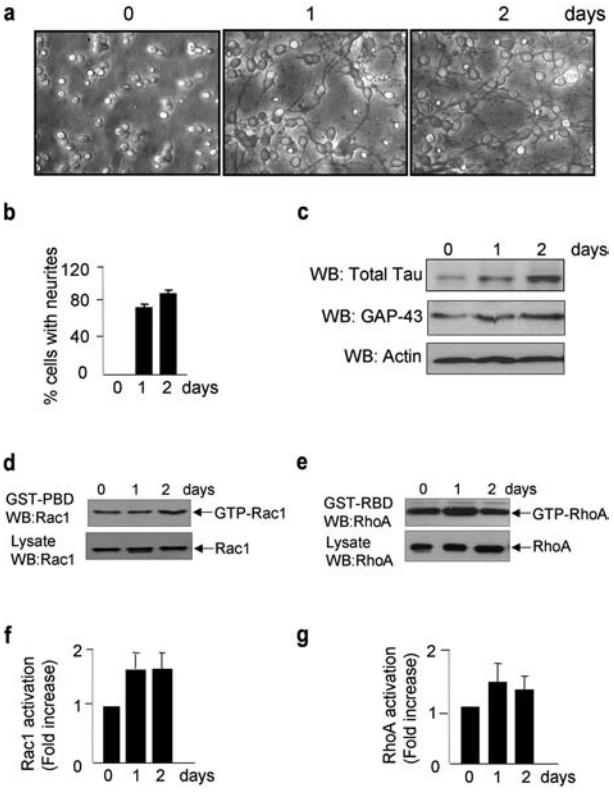

Figure 4. CGNs cultured under depolarizing conditions form neurite outgrowths. CGNs were grown *in vitro* for 2 days to study neurite formation. (*a*) Phase-contrast photomicrographs showing cultured CGNs at different time points (20× magnification). (*b*) Histograms showing the percentage of cells with neurites (calculated from ten randomly selected fields). (*c*) Western blots showing the expression of neuronal markers in CGNs cultured under depolarizing conditions. Total cellular proteins (50 µg) separated by SDS-PAGE were blotted and probed with antibodies against total tau and GAP-43. The blots were re-probed with anti-actin antibody to determine loading differences. (*d*, *e*) Activation of RhoA and Rac1 was examined as described in the legend of Fig. 2b. GTP-bound forms of Rac1 and RhoA were detected in CGNs; Rac1 and RhoA levels remained unchanged over 48 h in depolarizing conditions. (*f*, *g*) Histograms showing the level of Rac1/RhoA activation in CGNs over 2 days of culturing. Fold activation represent mean ± SD from neuronal preparation from five different litters.

cytoskeleton. Moderate BACs stunted neurite outgrowth (Fig. 1c). Although we observed very small neurites in CGNs from unexposed control pups, we observed few if any neurites in CGNs isolated from ethanol-exposed pups (Fig. 1c). MTT assay further confirmed that moderate BACs were non-toxic (Fig. 1d). Ethanol exposure failed to affect the formation of focal adhesions (Fig. 1e, f).

Distinct neurites in primary CGN cultures were present after 24 h. To determine whether moderate concentrations of alcohol affect neurite extension, we incubated CGNs isolated from ethanol-exposed pups in the presence of 183 mg/dL or ~40 mM ethanol. Exposure to ethanol stunted neurite formation in approximately 40% of CGNs (Fig. 1 g, h), and, of the CGNs that did form neurites, these had few secondary branches (Fig. 1i, j). These studies indicated that neurite formation was more sensitive to ethanol than was cell survival.

CGNs from ethanol-exposed pups show Rac1 inhibition and RhoA activation. Rho GTPases, RhoA and Rac1, are regulators of cytoskeletal rearrangement and are necessary during neurite formation. Hence, we studied the effect of ethanol on the activation of these GTPases. Moderate BACs resulting from 3 h of exposure to ethanol vapors inhibited Rac1, as indicated by the low signal intensity of GTP-Rac1 (Fig. 2a, b). Activation of RhoA, however, was unaffected by this ethanol concentration (Fig. 2a, c).

Because ethanol induces apoptosis in CGNs *in vitro* [19], we wondered whether exposing pups to ethanol for longer than 3 h induces apoptosis as well as raises BAC. Indeed, exposing pups to ethanol-saturated air for 5 h not only increased BAC (384.75 ± 52.6 mg/dL or ~80 mM) to levels higher than those resulting from 3 h of ethanol exposure, but also induced apoptosis in the cerebellar tissue, as demonstrated by TUNEL staining (Fig. 3a, b). Next, we assessed the viability of CGNs isolated from ethanol-exposed pups using a MTT assay. CGNs from ethanol-exposed pups formed reduced levels of formazan, indicating that these neurons had diminished viability (Fig. 3c). To determine whether ethanol affected RhoA and Rac1 signaling, we examined the activation of RhoA and Rac1 in CGNs isolated from pups exposed to ethanol for 5 h. Pull-down assays revealed that ethanol exposure inhibited Rac1 activation, as indicated by a weak GTP-Rac1 signal in CGNs from ethanol-exposed pups relative to that in CGNs from unexposed pups (Fig. 3d, e). By contrast, ethanol exposure increased RhoA activation, as indicated by a strong GTP-RhoA signal in the CGNs from ethanol-exposed pups (Fig. 3e).

Rac1 and RhoA regulate neurite outgrowth formation in CGNs. To confirm the role of Rac1 and RhoA in CGN differentiation, we examined neurite formation and Rac1 and RhoA activation in CGNs cultured under depolarizing conditions. Neurites began to form 12–15 h after plating; distinct neurites were observed after 2 days of *in vitro* culturing (Fig. 4a, b). Neurite formation was accompanied by increased expression of neuronal marker proteins GAP-43 and total tau (Fig. 4c). Neurons that failed to form neurites subsequently separated from the plates. By the end of 2 days of culturing, almost all neurons were interconnected by a network of neurites.

We examined RhoA and Rac1 activation in CGN cultures, starting 2 h after plating and continuing to 2 days, a time corresponding to neurite formation (day 0 represents the earliest point after attachment of the cells to the culture dish). We detected active (GTP-bound) forms of Rac1 and RhoA in CGNs cultured for 2 days (Fig. 4d–g).

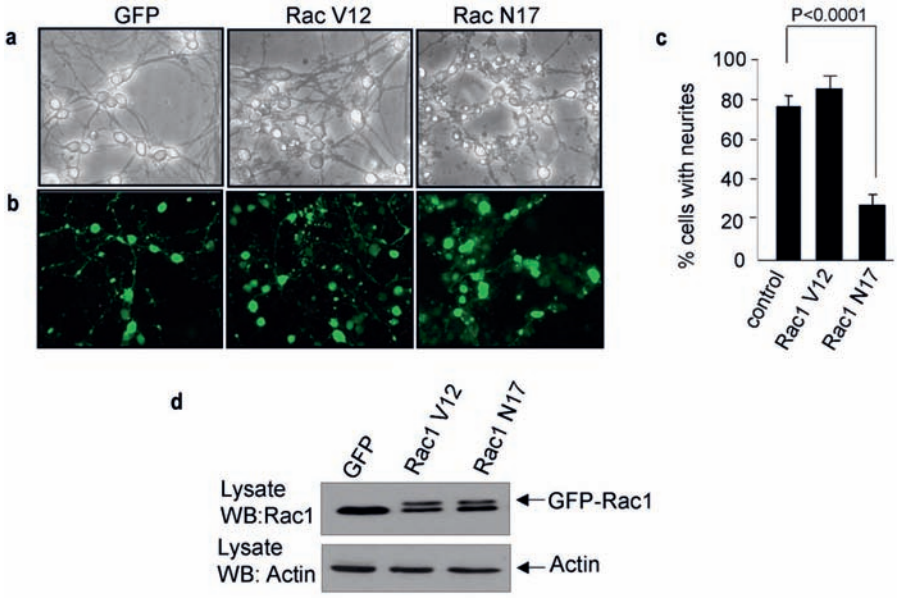

Figure 5. Inhibition of Rac1 blocks induction of neurite outgrowth. CGNs were grown *in vitro* for 24 h and then infected with adenovirus expressing either dominant negative Rac1 (Rac1 N17) or constitutively active Rac1 (Rac1 V12) at an moi of 100 for 48 h. (*a*) Phase-contrast photomicrographs of CGNs showing the inhibition of neurite formation after expression of Rac1 N17 (20× magnification). (*b*) Fluorescence photomicrographs showing the distribution of GFP-Rac1 expressed in CGNs (20× magnification). (*c*) Comparison of Rac1 V12-expressing CGNs and Rac1 N17-expressing CGNs bearing neurites. We counted the number of CGNs with neurites from ten random fields from three different experiments. Some CGNs expressing Rac1 N17 had shrunken cell bodies, suggesting that they may have been undergoing apoptosis. (*d*) Western blots showing the expression of GFP-Rac1 V12 and GFP-Rac1 N17 in CGNs. Lower bands represent endogenous proteins and upper bands represent overexpressed proteins. Blots were re-probed with anti-actin antibody to check loading differences. Results are mean ± SD from three different experiments.

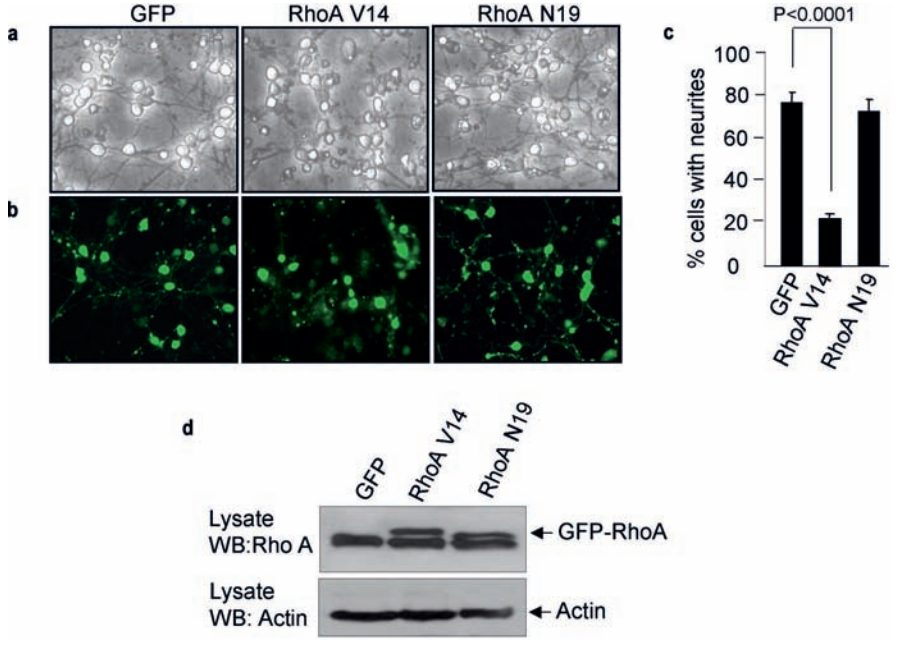

Figure 6. Activation of RhoA blocks neurite formation. CGNs were grown *in vitro* for 24 h and infected with adenoviruses expressing constitutively active RhoA (RhoA V14) or dominant negative RhoA (RhoA N19) at an moi of 100 for 48 h. (*a*) Phase-contrast photomicrographs of CGNs showing the inhibition of neurite formation after expression of RhoA V14 (20× magnification). (*b*) Fluorescence photomicrographs showing the distribution of GFP-RhoA expressed in CGNs (20× magnification). (*c*) Comparison of RhoA V14-expressing CGNs and RhoA N19-expressing CGNs bearing neurites. Percent cells with neurites were evaluated from ten random fields. Results are mean ± SD from three different experiments. (*d*) Western blots showing the expression of GFP-RhoA V14 and GFP-RhoA N19 in CGNs. Lower bands represent endogenous proteins and upper bands represent overexpressed proteins. Blots were re-probed with anti-actin antibody to check the loading differences. Results are mean ± SD from three separate experiments.

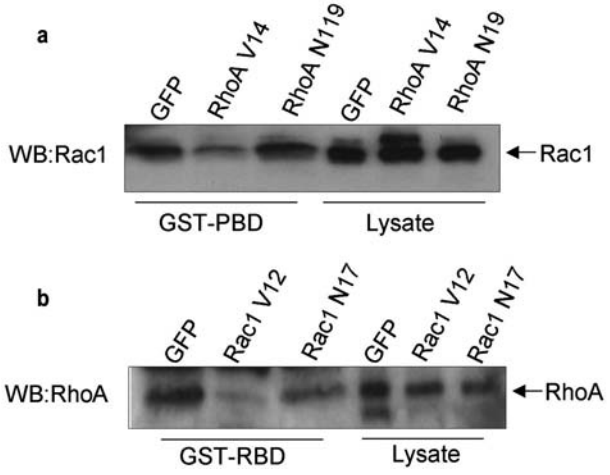

Figure 7. RhoA and Rac1 function in an antagonistic manner. Antagonistic action between RhoA and Rac1. (*a*) RhoA V14 blocked Rac1 activation. CGNs were infected with adenovirus expressing RhoA V14 or RhoA N19, and Rac1 activation was studied as described in the legend for Fig. 2b. (*b*) Rac1 V12 blocked RhoA activation. CGNs were infected with adenovirus expressing either Rac1 V12 or Rac1 N17, and RhoA activation was studied by GST-RBD pull-down assay (see legend for Fig. 2b). Results are mean ± SD from three separate experiments.

Rac1 and RhoA activation remained unchanged for up to 5 days of culturing (data not shown).

To further elucidate the role of RhoA and Rac1 in neurite formation, we infected CGNs with adenoviruses expressing either constitutively active or dominant negative forms of RhoA and Rac1. Overexpression of the constitutively active (V12) and dominant negative (N17) Rac1 remained unaltered for 48 h (Fig. 5a–d). Neurite formation was inhibited in cells expressing Rac1 N17, indicating that GTP-Rac1 is involved in neurite formation (Fig. 5c).

Mutated forms of RhoA were similarly expressed in CGNs (Fig. 6a–d). Overexpression of RhoA V14 inhibited neurite formation, while overexpression of RhoA N19 had no effect (Fig. 6c). Overexpression of constitutively active RhoA inactivated Rac1 (Fig. 7a), while overexpression of Rac1 V12 blocked activation of RhoA (Fig. 7b). Taken together, these findings suggest that, in a functional sense, an antagonistic relationship exists between RhoA and Rac1 in CGNs.

Discussion

Ethanol is a potent neurotoxic agent, and exposure to ethanol during development results in fetal brain defects, with the cerebellum being one of the major areas affected [46–49]. In the present study, we examined the effects of moderate and high ethanol concentrations on differentiation of CGNs. These concentrations are comparable to blood alcohol levels observed in intoxicated individuals [50]. Ethanol-tolerant individuals can have BACs of up to 540 mg/dL (~120 mM) [9], and pregnant women can have BACs as high as 375 mg/mL (~80 mM) [51]. Thus, it is conceivable that fetuses of mothers who drink could be exposed to similar ethanol concentrations used in our study. Ethanol induces cell death in both Purkinje cells and CGNs, the two major cell types in the cerebellum [19, 52, 53]. Since the susceptibility of rat Purkinje cells to ethanol-related damage decreases at P7, in the present study we focused on the effects of ethanol on CGNs, which are undifferentiated and remain susceptible at P7 [22, 23]. Exposure to moderate ethanol concentrations was not apoptotic but inhibited neurite formation as well as secondary branching. A similar inhibitory effect of ethanol on neurite formation and branching has also been observed during L1-induced neurite formation in CGNs, nerve growth factor-induced neurite formation in PC12 cells and cortical neurons, and retinoic acid-induced differentiation of neuroblastoma cells LA-N-5 and cortical neuroepithelial precursors [29, 54–58]. Our observations that moderate ethanol concentrations inhibit neurite formation in CGNs show that neurite formation is more sensitive to ethanol toxicity than is cell survival, an observation also reported in hippocampal and dorsal root ganglionic neurons [59, 60]. These observations are consistent with our findings that moderate concentrations of ethanol also inhibited activation of Rac1, a positive regulator of neurite formation [61]. Rac1, through activation of PAK1, brings about activation of LIMK and inhibition of cofilin to stabilize polymerized actin during lamellipodia and filopodia formation [62].

We found that higher ethanol concentration simultaneously induced activation of RhoA and inhibition of Rac1 in CGNs. RhoA is known to be involved in apoptotic cell death [63, 64]. In astrocytes, ethanol-induced activation of RhoA mediates apoptosis [65]; in spinal cord and PC12 cells, inactivation of RhoA by its antagonist protects cells from apoptosis [66, 67]. It is possible that ethanol-induced activation of RhoA might be responsible for apoptosis in CGNs. However, studies performed *in vitro* using adenovirus containing the gene encoding constitutively active form of RhoA (Fig. 6) suggested that activation of RhoA alone might not be responsible for induction of cell death in CGNs. It might be responsible for neurite retraction as suggested by lesser number of neurites formed due to the expression. How ethanol activates RhoA is unclear. In a recent study, phosphorylation of Rho GDI by Src prevented its complex formation with RhoA [68]. Although not reported so far, it is possible that ethanol at higher concentration activates Src, which phosphorylates RhoGDI and disinhibits RhoA. Similar types of signaling events might also be involved in activation of other components for loading of GTP and activation of RhoA [69].

The observation that ethanol also inhibited Rac1 in CGNs at higher concentration is in accordance with the pro-survival role of Rac1 in CGNs [70]. In human lymphoma cells, Rac1 inhibits apoptosis through phosphorylation of Bad [71], whereas in NIH3T3 and 293T cells, Rac1 inhibits apoptosis through activation of pro-survival kinase Mirk [72]. Recently, caspase-mediated cleavage of Rac1 has also been observed during apoptotic death in CGNs [70]. Ethanol-induced cell death in cerebellum has also been shown to be accompanied by altered insulin-IGF-I/IGF-II signaling [73]. IGF-I is known to activate phosphatidylinositol 3-kinse/Rac1 pathway [74], and plays a pro-survival role in CGNs [75]. These studies highlight the possibility that ethanol might also impair IGF/Rac1 signaling pathway for affecting survival of CGNs. Differential regulation of Rho GTPases by ethanol that results in simultaneous activation of RhoA, and inhibition of Rac1, might be contributing to the induction of apoptosis in CGNs.

We also confirmed the dominant role of Rac1 in regulating neurite formation in CGNs by growing them under depolarizing conditions that cause them to form neurites and to increase expression of GAP-43 and total tau proteins. These proteins are involved in microtubule polymerization and stabilization in growing neurites, and thus their increased expression indicates maturation of CGNs over time [76–78]. The Rho family GTPases, RhoA and Rac1, become activated in response to neurotrophic stimuli to regulate the microtubule assembly/disassembly necessary during neurite formation [43, 79–81]. Various neurotrophic factors, such as semaphorins, ephrins, netrins, and neurotrophins, activate Rho GTPases during axonal growth [82–85]. In the case of CGNs, the depolarizing conditions we used during *in vitro* culturing may have provided a stimulus for activating RhoA and Rac1. The regulatory role of Rac1 in neurite formation in cell lines as well as primary neuronal cultures has been reported [43, 82, 86–88]. Rac1, which generally localizes to the tips of growing neurites, is involved in actin polymerization, while active RhoA, which localizes to the distal end of growth cones, brings about actin de-polymerization [30]. Thus, taken together with our observations, Rac1 activation may be involved in regulating neurite outgrowth in CGNs. Although Rac1 V12 expression induces neurite formation [43], we did not observe Rac1 V12-enhanced neurite outgrowth in our preparations. Rac1 V12-associated outgrowth may have been masked by the neurite outgrowth caused by culturing CGNs under depolarizing conditions, since these conditions also induce neurite outgrowth in CGNs. On the other hand, we observed that RhoA V14 expression inhibited neurite formation perhaps by inhibiting Rac1, which has a dominant role in neurite formation. Indeed, we observed an antagonistic effect between RhoA and Rac1. The opposing actions of RhoA and Rac1 on neurite formation is well documented, and is facilitated by differential compartmentalization of active RhoA and Rac1 within the cell [30]. The balance between RhoA activation and Rac1 activation plays a critical role during neurite formation, and such a balance might exist in CGNs [82, 89].

In conclusion, our studies show that Rac1 has a dominant role in regulating neurite formation in CGNs cultured under depolarizing conditions. Under moderate conditions, ethanol affects CGN differentiation by inhibiting neurite process formation and Rac1 activation. On the other hand, higher ethanol concentrations induce apoptotic cell death along with RhoA activation and Rac1 inhibition. Ethanol thus appears to have a dual effect on the developing cerebellum: higher concentrations promote apoptosis, while lower concentrations inhibit differentiation.

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