Chronic ethanol exposure alters the levels, assembly and cellular organization of the actin cytoskeleton and microtubules in hippocampal neurons in primary culture

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Running Title: Ethanol alters actin and tubulin in neurons
Abstract

The organization and dynamics of microtubules and the actin cytoskeleton are critical for the correct development and functions of neurons, including intracellular traffic and signaling. *In vitro* ethanol exposure impairs endocytosis, exocytosis and nucleocytoplasmic traffic in astrocytes, and alters endocytosis in cultured neurons. In astrocytes, these effects relate to changes in the organization and/or function of microtubules and the actin cytoskeleton. To evaluate this possibility in hippocampal cultured neurons, we analyzed if chronic ethanol exposure affects the levels, assembly, and cellular organization of both cytoskeleton elements and the possible underlying mechanisms of these effects by morphological and biochemical methods. In the experiments described below, we provide the first evidence that chronic alcohol exposure decreases the amount of both filamentous actin and polymerized tubulin in neurons, and that the number of microtubules in dendrites lowers in treated cells. Alcohol also diminishes the microtubule-associated protein-2 levels, which mainly localizes in the somatodendritic compartment in neurons. Ethanol decreases the levels of total Rac, Cdc42 and RhoA, three small GTPases involved in the organization and dynamics of the actin cytoskeleton and microtubules. Yet when alcohol decreases the levels of the active forms (GTP-bound) of Rac1 and Cdc42, it does not affect the active form of RhoA. We also investigated the levels of several effector and regulator molecules of these GTPases to find that alcohol induces heterogeneous results. In conclusion, our results show that microtubule, actin cytoskeleton organization and Rho GTPase signaling pathways are targets for the toxic effects of ethanol in neurons.

Key words: Neurons, chronic ethanol, actin, tubulin, MAP2, Rho GTPases
INTRODUCTION

Clinical and experimental evidence indicates that alcohol (ethanol) consumption during pregnancy disrupts the developmental processes in the CNS, leading to the depression of neurogenesis, delayed and aberrant neuronal migration, and anomalous development (Goodlett and Horn, 2001). Thus, the offspring of women who drink alcohol during pregnancy may be affected either by alcohol-related neurodevelopmental disorders (ARND) or fetal alcohol syndrome (FAS), the most common preventable causes of mental retardation (Sampson et al., 1997). No single mechanism has proved sufficient to account for these effects, and it is likely that multiple factors are involved (Goodlett et al., 2005; Martínez and Egea, 2007). One such mechanism suggests that ethanol perturbs protein trafficking (Marín et al., 2008, 2010; Megías et al., 2000; Tomás et al., 2002, 2004, 2005). In astrocytes, these effects have been attributed to a primary alteration of the cytoskeleton (Guasch et al., 2003; Martínez et al., 2007; Renau-Piqueras et al., 1989; Tomás et al., 2003, 2005). The effects of chronic alcohol exposure in impairing endocytosis in cultured neurons were accompanied by alterations of several proteins involved not only in the endocytic process, but also in the organization of the actin cytoskeleton, suggesting that chronic alcohol exposure affects this cell component in these cells (Marín et al., 2010).

The organization and dynamics of the actin cytoskeleton and microtubules (MT) are critical for the correct development and function of neurons (Govek et al., 2005; Hoogenraad and Bradke, 2009), and are regulated by several GTPases of the Rho family of which the best studied are RhoA, Rac1 and Cdc42 (Iden and Collard, 2008; Heasman and Ridley, 2008). In addition, these GTPases play a key role in the regulation of several aspects of neuronal development, including the establishment of neuronal...
polarity, neurite outgrowth and differentiation, axon pathfinding, dendrite initiation, growth and branching, as well as dendritic spine formation and maintenance (Calabrese et al., 2006; Linseman and Loucks, 2008; Nakayama et al., 2000). The Rho GTPases cycle is regulated by three classes of proteins (Iden and Collard, 2008): GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins) and GDIs (guanine nucleotide dissociation inhibitors). These Rho GTPase regulators not only modulate Rho GTPase activity, but often define downstream signaling pathways by mediating the interactions between Rho GTPases and specific effector molecules (Iden and Collard, 2008; Govek et al., 2005). To date, numerous possible effectors for Rho, Rac and Cdc42 and their implication in actin and MT dynamics have been identified. Some of these effectors are scaffold proteins, whereas others possess kinase activity (Iden and Collard, 2008; Govek et al., 2005).

The normal cognitive function depends on adequate neuronal differentiation and migration, proper axonal pathfinding and the correct formation of synapses at contact sites on dendritic spines. These processes are regulated by Rho GTPase signaling pathways. Therefore, the deregulation of these pathways is implicated in several pathological conditions, including neurodegenerative disorders and some forms of mental retardation (Govek et al., 2005; Linseman and Loucks, 2008). Currently, it is thought that mutations in the Rho-linked genes could result in mental retardation by disrupting the development, structure, and/or plasticity of neuronal networks via perturbations in the regulation of the actin cytoskeleton (Ramakers, 2002). Along these lines, previous studies into astrocytes have suggested that alterations in the structure and organization of MT and the actin cytoskeleton could be involved in alcohol-induced alterations in the developing brain (Guasch et al., 2003; Martínez et al., 2007; Tomás et al., 2003, 2005).
Studies into the effect of alcohol exposure on the neuronal cytoskeleton during differentiation and/or function are scarce, but some have indicated the possible relevance of this effect as an underlying alcohol toxicity mechanism on the brain (Ahluwalia et al., 2000; Depaz et al., 2005; Joshi et al., 2006; Marín et al., 2010; Sordella and Van Aelst, 2006). Here we provide new evidence to support this possibility. We analyzed if chronic ethanol exposure affects the following aspects of actin and MT organization and dynamics in rat-growing hippocampal neurons in primary culture: 1) amount, assembly and cellular organization of actin and MT, 2) the levels and distribution of the MT-associated protein-2 (MAP2), 3) the levels of the total and active forms of Rac1, Cdc42 and RhoA, 4) the level of several effectors for these GTPases. These include some of the best characterized Cdc42, Rac and RhoA effector proteins: IQGAP1; the Wiskott-Aldrich syndrome protein (WASP); the WASP family verprolin-homologous protein (WAVE); the p21-activated kinase (αPAK); and the mammalian Ena Enabled (Mena); all of which are effectors of Rac and/or Cdc42. As a RhoA effector, we analyzed diaphanous 1 (Dia), 5) the amount of GDI, GEF and GAP.
MATERIALS AND METHODS

Reagents

**Primary antibodies:** Anti-\(\alpha\) tubulin (B-7) mouse monoclonal antibody, anti-IQGAP1 (H-109) rabbit polyclonal antibody, anti-p21-activated kinase (\(\alpha\)PAK) (N-20) rabbit polyclonal antibody, anti-diaphanous 1 (Dia1 also designated p140mDia) (V-20) goat polyclonal antibody, anti-mammalian Ena Enabled (Mena) (21) mouse monoclonal antibody, anti-neural-Wiskott-Aldrich syndrome protein (N-WASP) (H-100) rabbit polyclonal antibody, anti-WASP family verprolin-homologous protein (WAVE1) (C-17) goat polyclonal antibody, anti-RhoGDI\(\alpha\) rabbit polyclonal antibody, anti-insulin receptor substrate p53 (IRSp53) (W-20) goat polyclonal antibody and anti-IRSp53 (C-16) goat polyclonal antibody were from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). Anti-\(\beta\)Pix (PAK-interacting exchange factor, also known as ARHGEF7 or Cool-1) rabbit polyclonal antibody and anti-microtubule-associated protein-2 (MAP2) rabbit polyclonal antibody were from Chemicon (Millipore Iberica S.A.U., Spain). Anti-cofilin rabbit polyclonal antibody was from Cytoskeleton (Denver, CO USA). Anti-actin-related protein 3 (Arp3) mouse monoclonal antibody, anti-RhoGAP rabbit monoclonal antibody, anti-acetylated \(\alpha\)-tubulin mouse monoclonal, anti-synaptosome-associated protein of 25 kDa (SNAP-25) and anti-postsynaptic density 95 (PSD-95) mouse monoclonal antibodies were purchased from Abcam (Cambridge, UK). Anti-neurofilament (NF) 200 rabbit polyclonal antibody and anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody were obtained from Sigma-Aldrich (Spain).

**Secondary antibodies:** Alkaline phosphatase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG were from Sigma-Aldrich.

Alexa Fluor® 594 chicken anti-rabbit IgG, and donkey anti-mouse rhodamine conjugated were obtained from Molecular Probes (Invitrogen S.A., Spain).
Other reagents: For the quantitative detection of RhoA, Cdc42 and Rac activity, the G-LISA small G-protein activation colorimetric-based assays were used (Cytoskeleton, Cat. Nos. BK124, BK127 and BK125, respectively). The ratio of G-actin to F-actin (G-actin/F-actin) and the ratio of tubulin to MT (MT/tubulin) were evaluated using two in vivo kits from Cytoskeleton (Cat. Nos. BK037 and BK038, respectively). While using these kits, protein determination was carried out using the Precision Red™ Advanced Protein Assay Reagent from Cytoskeleton (Cat. No. ADV02).

F-actin was also analyzed using FITC-labeled phalloidin (Sigma-Aldrich, Spain) and by fluorescence microscopy.

Primary Culture of Hippocampal Neurons and Alcohol Treatment

The primary cultures of hippocampal neurons were prepared from the fetuses of female rats on day 16 of gestation as described in detail (Marín et al., 2010). Cells were cultured in Neurobasal medium containing B27 and Glutamax, and maintained in a humidified atmosphere (5% CO₂ and 95% air) at 37°C. In this medium, glial cell growth at five days dropped to less than 1.0%, resulting in a nearly pure neuronal population (Price and Brewer, 2001; Marín et al., 2010). The medium was changed every 2 days and cells were maintained until day 14 (14 days in vitro or DIV). To analyze the possible toxic effects of alcohol on the cytoskeleton during neuronal development, some cells were grown in the presence of ethanol which was added to the culture medium on the day of plating. The ethanol concentration in the medium was checked daily and adjusted to a final concentration of 30 mM (138 mg/dl, ethanol evaporation after 24 h was 10-20%), similar to the blood levels reported in pregnant chronic drinkers and considered moderate alcohol consumption (Eckardt et al., 1998). The concentration of alcohol in the medium was determined using a kit from Sigma-Aldrich (NAD-ADH Reagent Multiple Test Vial N7160). The purity of neuron cultures was assessed by
immunofluorescence using anti-GFAP anti-MAP2, and anti-NF antibodies (Marín et al., 2010). The anti-SNAP-25 and anti-PSD-25 antibodies were used as pre- and post-synaptic markers (Marín et al., 2010). Our results indicate that cell cultures contained 97-99% of neurons and that both synaptic markers were present in most of the examined cells. Cell viability was determined by the trypan blue exclusion test. In some cultures, the possible cytotoxic effect of alcohol exposure on neurons was also evaluated using a cytotoxicity assay kit (Sigma-Aldrich, Cat. Num. TOX7). These tests indicate that excellent long-term survival was achieved in the control and alcohol-exposed cells after 2 weeks in culture with more than 95% viability.

All the experiments using rats were approved by the appropriate institutional review committee and performed in strict compliance with the European Community Guide for the Care and Use of Laboratory Animals.

G-Actin/F-Actin In Vivo Assay

The F-actin/G-actin ratio in cells was analyzed using an F-actin/G-actin in vivo assay kit following the manufacturer's protocol (Cytoskeleton). Cell homogenization and centrifugation were done at 37°C to maintain F-actin stability. Cells were washed with PBS, lysed with a cell lysis and F-actin stabilization buffer, and then homogenized using 25 G syringes. After 10 min of incubation in this buffer, cell lysates were centrifuged at 400 g for 5 min, and supernatants were centrifuged again at 100,000 g for 60 min. The supernatants containing G-actin were separated from the pellets (F-actin) and were immediately placed on ice. Pellets were resuspended in a volume equivalent to that of the G-actin supernatant using ice cold dH2O containing 10 µM cytochalasin D. They were incubated on ice for 60 min by mixing with 25 G syringes every 15 min. Equal volumes of diluted (1:10) samples were loaded in each lane and analyzed by
Western blotting with the anti-actin antibody provided by the manufacturer. Total actin was calculated as the sum of G- and F-actin.

**MT vs. Free Tubulin In Vivo Assay**

To isolate the free and polymerized fractions of tubulin from the control and alcohol-treated neurons, an MT/tubulin *in vivo* assay kit was used (Cytoskeleton). For this purpose, cell homogenization and centrifugation were done at 37°C to maintain MT stability. Neurons were washed with PBS, and were then harvested and lysed in a cell lysis MT stabilization buffer and homogenized using 25 G syringes. After a 10-min incubation in this buffer, neuronal lysates were centrifuged at 100,000 g for 30 min, and the supernatants containing soluble tubulin were separated from the pellets containing MT (polymerized tubulin plus MAPs) and cooled on ice. Pellets were dissolved in a volume of dH₂O containing 200 µM CaCl₂. This volume was similar to that of the supernatant fraction. Pellets were then dissociated by incubation on ice with frequent disaggregation with 25 G syringes for 1 h. The protein concentrations of both the supernatants and the pellet fractions were determined using the reagent recommended by the manufacturer. Equal amounts of protein were loaded per well on 10-20% gradient SDS-polyacrylamide gels, and Western blotting analyses were conducted using an anti-tubulin antibody provided by the manufacturer to determine the tubulin in the “free tubulin” and “polymerized tubulin” (MT) fractions. Total tubulin was calculated as the sum of the free and polymerized tubulin.

**Measurement of the GTP-bound Form (active) of RhoA, Cdc42 and Rac**

The RhoA, Cdc42 and Rac active forms were determined from both the control and alcohol-exposed neurons using the G-LISA™ RhoA, Cdc42 and Rac activation assay biochemistry kits (Cytoskeleton) according to the manufacturer’s instructions.
Briefly, protein was isolated using the cell lysis buffer provided by the manufacturer, and cells were processed rapidly on ice to preserve the active form, and snap-frozen at -70°C until assayed. After the lysis, neuron samples were clarified by centrifugation at 16,100 g at 4°C for 2 min. The protein concentration was determined according to the manufacturer’s protocol and the protein amount was adjusted for each GTPase assay.

**Fluorescence Microscopy**

The distribution of actin and MT in both the control and alcohol-exposed neurons was analyzed by fluorescence microscopy as previously described in astrocytes (Tomás et al., 2003). Cultured cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature, washed in PBS (3 x 5 min each) and in the PBS containing 50 mM ammonium chloride (10 min) to block any free aldehyde group. Subsequently, cells were permeabilized for 15 min with PBS containing 0.1% Triton X-100, 1% BSA and 10% FBS. Actin was visualized by incubating cells with 50 µg/mL FITC-labeled phalloidin in PBS containing 1% BSA for 60 min. For MT, cells were first incubated with a mouse anti-tubulin monoclonal antibody (1:50) and then with an anti-mouse antibody conjugated with rhodamine. In some cases double immunofluorescence experiments were also performed to demonstrate the actin and tubulin in the same cells. MAP2 distribution was also analyzed using an antibody anti-MAP2 (1:250) and a secondary anti-rabbit antibody (1:50) conjugated with Alexa 594 nm.

Finally, cells were rinsed several times in PBS and mounted in Mowiol. Microscopy and imaging were performed with either an Olympus BX50 epifluorescence microscope or a Leica SP5 confocal microscope. Images were processed in PC computers using the Adobe Photoshop CS3 program.
Western Blotting

The control and alcohol-exposed neurons were washed with PBS and homogenized in extraction buffer (6 mM Tris-buffer, 10 mM EDTA and 2% SDS, pH 7.0) with 5 µL/mL mammalian protease inhibitor cocktail. Then the cell lysates obtained were processed for Western blotting as previously described (Marín et al., 2010; Tomás et al., 2005). The proteins analyzed comprised: 1) the RhoGTPases Rac, Cdc42 and RhoA (total levels), 2) several of the best studied RhoGTPases effectors (Figure 1 and Table 1), such as the Rac/Cdc42 effectors IQGAP, αPAK, WAVE1, N-WASP and Mena and the RhoA effector Dia, 3) the protein Arp3 belonging to the Arp2/3complex, which is involved in actin polymerization, 4) cofilin, which is an actin depolymerizing protein, regulated indirectly by αPAK and, 5) the regulators Rho GDIα, Rho GAP and βPix. In addition to these proteins, the levels of MAP2 and acetylated-tubulin were also determined in the control and ethanol-exposed neurons. Gel quantification was conducted using the ImageJ program (version 1.43). Linearity ranges were established by blotting different amounts of each sample. The results are shown as the mean values of at least 4-6 different experiments per group.

Electron Microscopy

For the ultrastructural studies, the plastic culture dishes containing neurons were randomly selected and washed in PBS. Cells were fixed “in flat” with 1.5% glutaraldehyde + 1.0% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 60 min. After washing in this buffer, cells were postfixed in 2% O₃O₄ containing 0.8% potassium ferrocyanide in cacodylate buffer for 60 min. Neurons were then treated with 0.1% tannic acid in buffer for 1 min at room temperature, washed in buffer and stained in block with 2.0% aqueous uranyl acetate for 120 min at room temperature, dehydrated
in ethanol and, finally, embedded as monolayers in Epon 812 (Marín et al., 2010). For this procedure, cell monolayers were covered with a layer of 2-3 mm of Epon 812 and dishes were placed at 60°C for 48-72h until the resin had completely polymerized. The cell-containing fragments from the Epon dish were glued onto Epon blocks or clamped into a holder for ultramicrotomy (Bozzola, 1999; Deitch and Banker, 1993), and ultrathin sections were double counterstained with acetate uranyl (20 min) and lead citrate (1 min) to be examined at 80 kV in a Philips CM 100 Electron Microscope.

To quantify the relative density of MT in dendrites, transversal ultrathin sections from the monolayers of the control and alcohol-exposed neurons were used. Then 25 profiles of the cross-sectioned dendrites in each cell population were recorded at x 10,500. The profiles of the dendrites of both the control and alcohol exposed cells should be circular (axial ratio ~1) with a similar diameter, and should clearly display the cross-sectioned MT. Micrographs were analyzed with the ImageJ program and the area of each profile was determined. The numerical density (Na) of MT (MT/µm²) was calculated by dividing the number of MT (N) counted in a profile by the area (A) of this profile (Na = N/A). In addition, the mean diameter of a population of randomly selected cross-sectioned dendrites of both the alcohol-exposed (n=25) and control neurons (n=27) were also calculated using the area values determined by the ImageJ program.

Moreover, the mean interMT distance (r₀) was determined using the expression r₀ = 1/Na⁻¹/³ (Marín et al., 2008). To assess the distribution of MT on the dendrite profiles, micrographs were analyzed using a test for ‘clumping’ which consists in calculating the variance:mean ratio. For this, a simple quadratic test system (each quadrat measuring 32.4 µm²) was superimposed on randomly selected micrographs. The mean MT number per quadrat was estimated, and distribution over quadrats was evaluated using the
variance:mean ratio, $R$. If ratio $R$ was significantly greater than one, a clumped pattern was indicated (Williams, 1977; Renau-Piqueras et al., 1989).

To differentiate between axons and dendrites, we followed the criteria described previously for hippocampal cultures (Bartlett and Banker, 1984), which indicate that unmyelinated axons can be as thin as 0.1 to 0.15 $\mu$m, whereas the diameter of most distal segments of dendrites ranges from 0.5 to 1.0 $\mu$m.

Statistical Analysis

The results are expressed as mean ± SD. For statistical computation and estimation of significance, we used the online GraphPad software (GraphPad Software, www.graphpad.com). Statistical significance was accepted when we obtained a p-value of < 0.05 using the Student’s t-test.
RESULTS

**Morphological Analysis of the Distribution of Actin, Tubulin and MAP2 in Neurons**

The distribution of actin, tubulin and MAP2 in the control and alcohol-exposed neurons was first analyzed by immunofluorescence techniques (Figs. 2 and 3). In these preparations, both cell types clearly showed stained soma, dendrites and axons when anti-tubulin or phalloidin were used. In addition, dendritic spines were also observed in some cells (Fig. 2). Moreover, the typical immunolabeling distribution of MAP2, a marker for dendrites, is shown in Figure 3. No apparent effects of alcohol exposure on the staining pattern of the three cytoskeletal proteins were observed. MAP2 levels were also analyzed by Western blotting. The results are summarized in Figure 3C and indicate that alcohol lowers the levels of this protein.

Transmission electron microscopy allows MT distribution in soma and neuronal processes to be qualitatively and quantitatively analyzed. However, this technique does well resolve microfilaments of actin. Figure 4 illustrates how alcohol induces changes, mainly in the morphology of MT. Thus MT frequently displayed an undulating shape in the soma of treated cells (Fig. 4D). These wavy MTs were virtually absent in the neuronal processes.

In the cross-sections of dendrites, we observed how alcohol treatment resulted in a sharp drop in the density of the MT profiles (Fig. 5). The quantitative analysis indicates that whereas the dendrites from the control neurons contained $94.04 \pm 32.93 \text{ MT/\mu m}^2$, the density of MT in treated cells dropped to $52.00 \pm 25.57 \text{ MT/\mu m}^2$ ($p < 0.0001$, Student’s t-test). Moreover, since the mean intraMT ($r_0$) distance was calculated as a function of MT density, we found that this parameter significantly increased in the alcohol-exposed neurons ($24.21 \pm 9.37 \text{ nm}$ in the controls and $40.64 \pm 20.44 \text{ nm}$ in the...
treated cells, p < 0.0001, Student’s t-test). In addition, the MT distribution in the cross-
sections of the treated neurons’ dendrites was heterogeneous. As Figure 5 depicts,
whereas MT appears to be homogeneously distributed in some profiles (Fig. 5C), these
structures accumulate asymmetrically on one side of the dendrite in others (Fig. 5D).
This tendency of MT distributing in the groups in some dendritic profiles was quantified
by a clumping test. Most of the randomly selected control profiles population showed
an MT distribution with R near to 1 (R= 1.1; total number of quadrats counted = 137.
The R of the section depicted in Figure 5B is 0.99). This indicates that the variance and
mean were practically equal, which is a characteristic of the Poisson distribution. Since
a value greater than one indicates a clumped pattern, the results of the treated neurons
indicate a clumping tendency for the whole population (R=1.8; total number of quadrats
counted = 139). As mentioned above, it is important to note that treated cells showed a
heterogeneous population of dendritic profiles with R-values ranging from 0.83 (Fig.
5C) to 3.4 (Fig. 5D). On the other hand, no differences between the mean dendrite
diameters of the control (0.74 ± 0.22 µm) and alcohol-exposed (0.73 ± 0.21 µm) cells
were found. These results of the diameter of the dendrites in both the control cells and
those relating to MT density are in agreement with previous studies (Bartlett and
Banker, 1984).

*Ethanol Affects the Ability of Actin and Tubulin to Polymerize*

In order to analyze the possible effect of ethanol on the ability of both tubulin
and actin to polymerize, an assay kit was used. The results obtained clearly indicate that
chronic alcohol lowered the total amount of actin (~13%) (Fig. 6C), diminished the F-
actin fraction, but did not induce significant changes in the G-actin fraction (Fig. 6A).

When analyzing the effect of alcohol on the levels of unpolymerized and
polymerized tubulin, this treatment was seen to clearly lower the total amount of tubulin
(-18%) (Fig. 6C), which results from an alcohol-induced lessening of both fractions, mainly that corresponding to MT (Fig. 6B).

Acetaldehyde, the first product of ethanol metabolism, formed covalent adducts with several proteins, including tubulin, after chronic ethanol exposure. The most important consequence of tubulin acetylation is that its polymerization was impaired; however it hyperstabilized once MT were formed (Shepard and Tuma, 2010; Yoon et al., 1998). Therefore, we analyzed if alcohol exposure changes the levels of acetylated tubulin in neurons. However, we found similar levels of this protein in both the control and ethanol-exposed cells (data not shown).

**Alcohol Exposure Decreases the Levels of the Active Forms of Rac and Cdc42, but does not Affect RhoA**

The organization and dynamics of the MT and actin cytoskeleton are regulated by several GTPases of the Rho family of which the best known are RhoA, Rac and Cdc42. GTPases cycle between active GTP-bound and inactive GDP-bound states. We used a procedure developed to quantify the levels of the active (GTP-loaded) forms of RhoA, Rac and Cdc42. As Figure 7 depicts, alcohol significantly decreases the levels of active Rac and Cdc42, but has no significant effect on the active form of RhoA.

In the Table inserted into Figure 7 (7D), we summarize the levels of total Rac, Cdc42 and RhoA (the results for the last two GTPases were previously published in Marín et al., 2010). As shown, alcohol treatment significantly lowered all these GTPases. However, since the levels of the active form of RhoA did not change after alcohol exposure, and alcohol lowered the amount of total protein shown in Figure 7D, it is possible to conclude that this treatment lowers the amount of the inactive form of this GTPase.
Effects of Ethanol Exposure on the Levels of Several GTPases Effectors, Regulators and Other Related Proteins

In addition to analyzing the effect of alcohol on the amount and active forms of the small GTPases RhoA, Cdc42 and Rac, we also did a Western blot to study the effect of this treatment on the level of the GTPase effectors WAVE1, N-WASP, Mena (140 and 80 kDa isoforms), αPAK and IQGAP1 (Fig. 1 and Table 1). We found that alcohol led to a dramatic drop in the levels of αPAK and IQGAP1 (Table 1). Moreover, we analyzed the levels of IRSp53 and Dia1 using several commercial antibodies, but no results were obtained. It is possible that IRSp53 is not expressed in neurons of 14 DIV (Choi et al., 2005).

The effect of chronic alcohol exposure of regulators RhoGDIα, RhoGAP and βPix, of which βPix belongs to the GEF family, was also investigated to find that only RhoGAP was affected (Table 1). The level of this GAP lowered by approximately 42% in the treated cells when compared to the control cells.

Finally, we evaluated the effect of alcohol on two proteins, cofilin and Arp3, which relate to actin polymerization and depolymerization (Govek et al., 2005). Whereas the levels of cofilin did not vary after alcohol exposure, Arp3 levels decreased by 70% in the treated cells (Table 1).
DISCUSSION

Chronic alcohol consumption negatively affects the adult and developing CNS, although the mechanisms involved in alcohol-induced toxicity in nervous cells remain unclear. Membrane traffic is critical during neural development and functioning, and one of the mechanisms proposed to explain these effects of alcohol, particularly in astrocytes, could be based on an alteration of cytoskeleton and intracellular trafficking (Marín et al., 2008; Megías et al., 2000; Tomás et al., 2004, 2005). Moreover, the recently described deleterious effect of alcohol on endocytosis in cultured neurons (Marín et al., 2010) could also be due to changes in the organization and/or function of MT and the actin cytoskeleton. Therefore in order to evaluate this possibility in neurons, we analyzed if chronic ethanol exposure affected the levels, assembly and cellular organization of both cytoskeleton elements. Regarding MT, ethanol reduced the amount of total tubulin if compared to the controls. In addition, we also found that the levels of polymerized tubulin from the ethanol-exposed neurons significantly reduced. These results show, for the first time, that a significant amount of tubulin in neurons is sensitive to chronic ethanol exposure, and suggest that this treatment alters the dynamic equilibrium between free and polymerized tubulin. Our results also indicate that ethanol alters the G-actin/F-actin ratio. To our knowledge, no previous studies have analyzed the levels of both forms of actin in nervous cells after chronic ethanol exposure. In addition to these biochemical assays, we conducted studies designed to determine whether alcohol alters the morphology and distribution of MT and actin microfilaments. Fluorescence microscopy confirmed the staining pattern of actin, tubulin and MAP2 in hippocampal neurons such as those described in previous studies (Akum et al., 2004; Kaech et al., 2001; Rapoport et al., 2002; Yanni and Lindsley, 2000). On the other hand, although an electron microscopic analysis of the neuronal actin filaments turned
out to be uninformative, this technique proved useful in the study of neuronal MT, mainly in the processes (Deitch and Banker, 1993; Peters et al., 1970). By using quantitative and qualitative procedures, we found that the number of MT in the cross-sections of the dendrites in the ethanol-exposed neurons significantly decreased when compared with the controls, and that this treatment also altered the distribution and morphology of these elements in soma and dendrites. Both the actin cytoskeleton and MT play an important role in maintaining the organization and function of dendrites and dendritic spines; that is, the major sites of excitatory synaptic input (Calabrese et al., 2006; Gu et al., 2008; Jaworski et al., 2009). The number and morphological characteristics of spines have also been reported to be significantly affected in children and in experimental animals prenatally exposed to ethanol (Berman and Krahl, 1996; Chen et al., 2003; Smith and Davies, 1990; Stoltenburg-Didinger and Spohr, 1983). Exposure to alcohol in vitro also lowered the values of several morphometric parameters of dendrites in rat hippocampal neuron cultures (Yanni and Lindsley, 2000). Therefore, it is reasonable to assume that these effects might be related to the alterations induced by ethanol given the basis of not only actin and tubulin’s ability to polymerize, but also the changes in the density and distribution of MT described in this study.

Changes in MT morphology may also be due to other effects of ethanol apart from those relating to tubulin’s ability to polymerize. We also analyzed the levels and distribution of MAP2 to find that alcohol affects these levels. Previous studies have reported that a long-term administration of alcohol decreases the MAP2 expression in the rat brain (Putzke et al., 1998). This protein is an important component of cross-bridge MT in dendrites and plays a key role in neurite initiation, MT stabilization, inhibition of depolymerization and increased MT rigidity (Dehmelt and Halpain, 2004). In addition, MAP2 has also been described to interact with actin microfilaments and...
neurofilaments by linking together the three cytoskeletal elements in dendrites (Dehmelt and Halpain, 2004; Chang and Goldman, 2004). Therefore, and as stated above, the alcohol-induced changes in MAP2 levels could be involved in the alterations to the morphology and stability of MT observed in the ethanol-exposed neurons.

Acetaldehyde adduction of tubulin as a result of ethanol metabolism may also be involved in the alterations to MT stabilization, structure and density which we observed in neurons after chronic alcohol exposure. To investigate this possibility, we used an antibody against acetylated tubulin which has been used in different cell types, including neurons (Toth et al., 2008). Levels of acetylated tubulin were equal in both the control and treated cells, suggesting that acetylation does not apparently play an important role in the alcohol-induced alterations to neuronal MT. Acetaldehyde has also been reported to form stable adducts in vitro with purified actin (Shepard and Tuma, 2010). However, whether this acetylation process occurs in vivo and the impact it may have on the actin cytoskeleton function in neurons remain to be shown.

Several studies in neural cells, including astrocytes and neurons (Guasch et al., 2003; Joshi et al., 2006; Martínez et al., 2007; Shaffert et al., 2006; Sordella and Van Aelst 2006), have identified small GTP binding proteins as targets of the effects of ethanol. However, the results described in neurons have been contradictory, which is likely due to the marked differences in the experimental designs, including ethanol doses and length of treatments. Thus, for example, whereas the GTP-active form of RhoA does not alter after chronic ethanol exposure (30 mM) for 14 days in our work, exposure (80 mM) of 7-day-old rat pups for 5 h inhibited Rac, but activated RhoA in cerebellar granule neurons (Joshi et al., 2006). In any case, as signaling by one or more small GTPases RhoA, Rac and Cdc42 is involved in dendrite initiation, growth and branching, neurite outgrowth and differentiation, and axon pathfinding, the alterations
induced by ethanol to any of these GTPases could alter these fundamental processes in neuronal biology.

The comparison of the results obtained in neurons with those found in astrocytes could be of interest because this comparison would allow a better understanding of some mechanisms by which ethanol affects RhoGTPases. Chronic alcohol exposure reduces endogenous levels of active RhoA in astrocytes in primary culture, but has no effect on the levels of active Rac or Cdc42 (Martínez et al., 2007). In contrast, we show herein that alcohol lowers the active forms and levels of Rac and Cdc42, but does not affect the active form of RhoA. Nevertheless, ethanol significantly decreases the total levels of RhoA, indicating that this treatment lowers the amount of GDP-RhoA (inactive form). What this suggests is that ethanol alters the shift in the GTP/GDP-bound RhoA equilibrium in neurons. Since one of the most important functions of the Rho family proteins is their role as regulators of actin cytoskeletal organization, the differential effects of alcohol on RhoGTPases in neurons and astrocytes could firstly be due to the presence of different forms of actin rearrangement in these cells. Thus, RhoA promotes the formation of actin stress fibers and focal adhesions and, while astrocytes show abundant stress fibers (Guasch et al., 2003; Martinez et al., 2007), these structures are not characteristic of neurons in vivo or in vitro. Moreover, the different effects of alcohol exposure on the three GTPases studied herein should also be analyzed from the perspective that the downstream effects of RhoA and Cdc42/Rac can be antagonistic to each another in several functional aspects in cells (Calabrese et al., 2006; Govek et al., 2005).

It remains to be clarified how the changes induced by ethanol to GTPases affect the amount of polymerized actin and tubulin in neurons. As mentioned earlier, the bridge between GTPases and cytoskeleton organization of both actin and MT is
established by effector and regulator molecules. Along these lines, we investigated the
effect of ethanol on several of the best characterized effectors of RhoGTPases (Fig. 1)
to obtain heterogeneous results. The effector proteins analyzed include IQGAP1, αPAK,
WAVE1 and N-WASP (Fig. 1). Alcohol treatment significantly lowers the levels of
IQGAP1 and αPAK which, together with the alterations induced by ethanol to Cdc42
and Rac, could alter the integrity and, therefore, the functionality, of actin cytoskeleton
and MT organization in alcohol-exposed neurons. IQGAP1, which plays a central role
in processes such as cell adhesion, polarization and migration, is an effector of Cdc42
and Rac, and contains binding sites for several molecules such as actin, Rac/Cdc42,
calmodulin or myosin, among others (Noritake et al., 2005). Moreover, by binding to
the MT tip protein CLIP170, IQGAP1 captures growing MT at the leading edge to
result in cell polarization. This interaction can be also regulated by signaling through
Cdc42 and Rac (Briggs and Sacks, 2003; Noritake et al., 2005). On the other hand,
PAKs are among the best-characterized effectors of Cdc42 and Rac. PAKs are a highly
conserved family of enzymes involved in the regulation of several cell functions such as
actin cytoskeleton dynamics, cell proliferation and differentiation, cell movement and
cell migration, and gene expression (Arias-Romero and Chernoff, 2008; Kreis and
Barnier, 2009). A deleterious effect of ethanol on PAK also could affect the other
functions of this effector relating with MT because PAK has several substrates involved
with MT disassembly (LIM kinase), stability (Op18/stathmin) or assembly of α/β-
tubulin heterodimers (tubulin cofactor B) (Szczepanowska, 2009).

In contrast to these results, the levels of WAVE1 and N-WASP, also related to
Cdc42 and Rac, do not change. Both proteins are fundamental actin cytoskeleton
reorganizers whose function is to receive upstream signals from Cdc42 and Rac and to
send them to activate the Arp2/3 complex, leading to rapid actin polymerization. This
complex comprises two actin-related subunits, Arp2 and Arp3, that mimic actin, bind to
the side of existing actin filaments and align in a similar manner to an actin dimer so
that the addition of a single actin monomer potently stimulates actin polymerization
(Korobova and Svitkina, 2008; Soderling, 2010). Although we observed no effects of
alcohol exposure on WAVE1 and N-WASP, it is interesting to note that this treatment
dramatically reduced Arp3 levels, which would result in a reduction of actin
polymerization in treated cells, among other effects.

On the other hand, we found no effects of alcohol on regulators Rho GDIα and
RhoGEF (βPix), whereas the level of Rho GAP, which is responsible for the
inactivation of RhoGTPases, lowered. Conversely, previous studies in astrocytes have
revealed that chronic ethanol exposure reduces the pool of GTP-bound RhoA, increases
RhoGAP, but does not alter the levels of RhoGEF (Martínez et al., 2007).

In conclusion, in this work hippocampal neurons from embryonic rat brain were
chronically exposed to moderate doses of ethanol. This treatment was initiated on the
day of plating, and was maintained until day 14 when cultures were mainly composed
of mature neurons. During this period, many important processes relating with neuronal
maturation occurred, including the formation of axons, dendrites, synaptogenesis and
dendritic spine morphogenesis. As different authors state, it must be understood that
although neuronal development in vitro is not a recapitulation of in vivo neuronal
maturation, the ontological progress of neurons in both situations is driven by common
stage-specific events (Chilton and Gordon-Weeks, 2006; Lesuisse and Martin, 2002;
Ringler et al., 2008). Most of these processes depend not only on the integrity of the
actin and MT cytoskeletons, but also on the systems regulating both cytoskeleton
systems. Therefore, the alterations we have observed after alcohol exposure to the
levels, assembly, and the cellular organization of actin and MT, may relate with the
alcohol-induced effects on the neuronal morphology and function described not only in children and experimental animals prenatally exposed to ethanol, but also in neuronal cells cultured in the presence of ethanol. Moreover, since the actin cytoskeleton and MT also relate with many important functions in neurons, including intracellular trafficking and signaling, alterations in the actin or MT cytoskeleton as a result of alcohol exposure could also affect these processes, as with endocytosis (Marín et al., 2010). Finally, the deregulation of Rho GTPase signaling, as described herein, has been claimed to relate with the development of several mental diseases, including those resulting from prenatal exposure to alcohol (Calabrese et al., 2006; Govek et al., 2005).

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FIGURES

FIG. 1. The organization and dynamics of the actin cytoskeleton and MT are regulated by several GTPases of the Rho family, including RhoA, Rac and Cdc42. Activated GTP-bound Rho GTPases interact with specific effector molecules to transduce upstream signals. The key effectors of Rac, Cdc42 and RhoA are IQGAP1, αPAK, WAVE1, N-WASPS, Mena, and Dia. Both the N-WASP and WAVE effectors are linked to the actin cytoskeleton through their interaction with the Arp2/3 complex. Cofilin is an actin depolymerizing protein which is regulated indirectly by αPAK. The effect of chronic alcohol exposure on these proteins in hippocampal neurons was analyzed in this study (Based in Govek et al. 2005).

FIG. 2. Immunofluorescence showing the distribution of actin in the control (A) and ethanol-exposed neurons (B). The images in (C) and (D) correspond to the distribution of tubulin in the control (C) and ethanol-exposed cells (D). Bars represent 10 µm.

FIG. 3. Immunofluorescence images showing the MAP2 distribution in the soma and dendrites of the control (A) and alcohol-exposed neurons (B). The effects of alcohol exposure on the levels of MAP2 were also analyzed by immunoblotting (C). The data used in the statistical analyses are the mean ± SD of 4-6 independent experiments. Asterisks indicate significant differences (p < 0.05). Student’s t-test; bars represent 10 µm.

FIG. 4. Electron microscopy micrographs illustrating the arrangement and shape of MT in the soma of the control (A and B) and ethanol-exposed (C and D) neurons. In both the control and treated neurons, MT appears to be arranged in parallel (arrowheads) or
in radially arranged paths (arrows in C). In the cell body of the alcohol-exposed cells, MT also presents undulating forms (arrows in D). Bars represent 1 µm (A and C) and 500 nm (B and D).

FIG. 5. (A) Electron microscopy micrograph showing an ultrathin section of the soma of a control neuron and several circular cross-sectioned processes. In the cell body, several cisternae of a Golgi complex are shown. Cross-sections of dendrites from the control (B) and alcohol-exposed (C and D) neurons as seen in the ultrathin sections. As shown, the density of MT (C) in many dendritic profiles from neurons exposed to alcohol is lower than that observed in the control cells (B). The quantitative results are summarized in the text. In addition, dendrites were frequently found with an asymmetric distribution of MT (D) which was distributed into clumps. Bars represent 400 nm (A) and 200 nm (B-D).

FIG. 6. Ethanol induces changes in the organization of actin and tubulin in neurons in primary culture. In (A), the relative F-actin and G-actin concentrations are determined. Alcohol treatment resulted in a significant reduction of F-actin. In (B), the analysis of the tubulin levels in MT pellets (MT) and supernatants indicate marked differences between the control and ethanol-exposed neurons. As shown, a lower amount of MT isolated in the pellets from the ethanol-treated neurons compared with the control cells is observed. (C) Histograms showing the effect of alcohol on total levels of actin and tubulin. Total actin and tubulin levels were calculated as the sum of G- and F-actin and free and polymerized tubulin, respectively.

The data used in the statistical analyses are the mean ± SD of four to six independent experiments. Asterisks indicate significant differences (p < 0.05). Student’s t-test.
FIG. 7. The levels of active (GTP-loaded) RhoA (A), Rac (B) and Cdc42 (C) in both the control and alcohol-exposed neurons. In (D), the total levels of the three RhoGTPases analyzed, RhoA, Rac and Cdc42, are summarized (the results for Rac and Cdc42 have been previously reported in Marín et. al., 2010). The data used in the statistical analyses are the mean ± SD of 4-6 independent experiments. Asterisks indicate significant differences (p < 0.05). Student’s t-test.
Table 1. Effects of alcohol exposure (30 mM, 14DIV) on the levels of the several effectors, regulators and related proteins involved in cytoskeletal organization in neurons.

<table>
<thead>
<tr>
<th>Protein type and function</th>
<th>Related GTPase</th>
<th>Variation in relation to control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WAVE1</td>
<td>Rac1</td>
<td>0 (=)</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Cdc42</td>
<td>0 (=)</td>
</tr>
<tr>
<td>Mena</td>
<td>Cdc42</td>
<td>0 (=) *</td>
</tr>
<tr>
<td>αPAK</td>
<td>Rac1, Cdc42</td>
<td>– 54 (↓)</td>
</tr>
<tr>
<td>IQGAP1</td>
<td>Rac1, Cdc42</td>
<td>– 37 (↓)</td>
</tr>
<tr>
<td><strong>Regulators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rho GDIα</td>
<td></td>
<td>0 (=)</td>
</tr>
<tr>
<td>Rho GAP</td>
<td></td>
<td>– 42 (↓)</td>
</tr>
<tr>
<td>βPix</td>
<td></td>
<td>0 (=)</td>
</tr>
<tr>
<td><strong>Other proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cofilin</td>
<td>Rac1, Cdc42</td>
<td>0 (=)</td>
</tr>
<tr>
<td>Arp3</td>
<td>Rac1, Cdc42</td>
<td>– 70 (↓)</td>
</tr>
</tbody>
</table>

We did not detect the presence of the effector IRSp53 (Insulin Receptor Substrate 53) by Western blot analysis which agrees with previous studies carried in brain at different ages (Choi et al., 2005). In the case of Dia 1, we were unable to demonstrate this protein using commercial antibodies.

* No changes in the 140 and 80 kDa isoforms (Lanier et al., 1999) were observed.

References can be found in: Bishop and Hall (2000); Bustelo et al., (2007); Govek et al., (2005); Iden and Collard (2008).
FIG. 1. The organization and dynamics of the actin cytoskeleton and MT are regulated by several GTPases of the Rho family, including RhoA, Rac and Cdc42. Activated GTP-bound Rho GTPases interact with specific effector molecules to transduce upstream signals. The key effectors of Rac, Cdc42 and RhoA are IQGAP1, αPAK, WAVE1, N-WASP, Mena, and Dia. Both the N-WASP and WAVE effectors are linked to the actin cytoskeleton through their interaction with the Arp2/3 complex. Cofilin is an actin depolymerizing protein which is regulated indirectly by αPAK. The effect of chronic alcohol exposure on these proteins in hippocampal neurons was analyzed in this study (Based in Govek et al. 2005).

177x100mm (600 x 600 DPI)
FIG. 2. Immunofluorescence showing the distribution of actin in the control (A) and the ethanol-exposed neurons (B). The images in (C) and (D) correspond to the distribution of tubulin in the control (C) and ethanol-exposed cells (D). Bars represent 10 µm. 88x88mm (600 x 600 DPI)
FIG. 3. Immunofluorescence images showing the distribution of MAP2 in the soma and dendrites of
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MAP2 were also analyzed by immunoblotting (C). The data used in the statistical analyses are the
mean ± SD of four to six independent experiments. Asterisks indicate significant differences (p <
0.05). Student’s t-test; bars represent 10 µm.

109x40mm (600 x 600 DPI)
FIG. 4. Electron microscopy micrographs illustrating the arrangement and shape of MT in the soma of the control (A and B) and ethanol-exposed (C and D) neurons. In both the control and treated neurons, MT appears to be arranged in parallel (arrowheads) or in radially arranged paths (arrows in C). In the cell body of the alcohol-exposed cells, MT also presents undulating forms (arrows in D).

Bars represent 1 µm (A and C) and 500 nm (B and D).

116x91mm (600 x 600 DPI)
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141x90mm (600 x 600 DPI)
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The data used in the statistical analyses are the mean ± SD of four to six independent experiments. Asterisks indicate significant differences (p < 0.05). Student’s t-test.

130x139mm (600 x 600 DPI)
FIG. 7. The levels of active (GTP-loaded) RhoA (A), Rac1 (B) and Cdc42 (C) in both the control and alcohol-exposed neurons. In (D), the total levels of the three RhoGTPases analyzed, RhoA, Rac1 and Cdc42, are summarized (the results for Rac1 and Cdc42 have been previously reported in Marín et al., 2010). The data used in the statistical analyses are the mean ± SD of four to six independent experiments. Asterisks indicate significant differences (p < 0.05). Student's t-test.