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Research report

Direct effects of ethanol on neuronal differentiation: An in vitro analysis of viability and morphology

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ABSTRACT

The deleterious effects of ethanol (EtOH) on the brain have been widely described, but its effects on the neuronal cytoskeleton during differentiation have not yet been firmly established. In this context, our aim was to investigate the direct effect of EtOH on cortical neurons during the period of differentiation. Primary cultures of cortical neurons obtained from 1-day-old rats were exposed to EtOH after 7 days of culture, and viability and morphology were analyzed at structural and ultrastructural levels after 24-h EtOH exposure. EtOH caused a significant reduction of 73 ± 7% in the viability of cultured cortical neurons, by preferentially inducing apoptotic cellular death. This effect was accompanied by an increase in caspase 3 and 9 expression. Furthermore, EtOH induced a reduction in total dendrite length and in the number of dendrites per cell. Ultrastructural studies showed that EtOH increased the number of lipidic vacuoles, lysosomes and multilamellar vesicles and induced a dilated endoplasmatic reticulum lumen and a disorganized Golgi apparatus with a ring-shape appearance. Microtubules showed a disorganized distribution. Apposition between pre- and postsynaptic membranes without a defined synaptic cleft and a delay in presynaptic vesicle organization were also observed. Synaptophysin and PSD95 expression, proteins pre- and postsynaptically located, were reduced in EtOH-exposed cultures. Overall, our study shows that EtOH induces neuronal apoptosis and changes in the cytoskeleton and membrane proteins related with the establishment of mature synapses. These direct effects of EtOH on neurons may partially explain its effects on brain development.

1. Introduction

Alcohol drinking is one of the most serious threats to human health from prenatal development to adulthood. However, ethanol (EtOH) is the most widely used and abused drug among humans.

Prenatal exposure to EtOH results in fetal alcohol syndrome and a variety of alterations included in the fetal alcohol spectrum disorders. These disorders are characterized by growth retardation, facial dysmorphology and a wide range of neurobehavioral anomalies (Riley and McGee, 2005; Spadoni et al., 2007; Guerri et al., 2009) including a decrease in learning capacity and memory, attention deficits, motor dysfunction and sudden infant death (Guerri, 1998; Evrard 2010; OíLeary et al., 2013). In addition, morphological abnormalities are found in several brain regions including the corpus callosum, cerebellum, caudate nucleus, basal ganglia and neocortex, both in humans and experimental animal models (Fryer et al., 2012; Fakoya and Caxton-Martins, 2006; Guerri, 2002; Ramos et al., 2002). Alterations are also observed in the relationship between neurons and glial cells, as well as in transcription factor expression (Evrard et al., 2003; Aronne et al., 2008, 2011). Although the mechanisms by which alcohol exposure exerts deleterious effects on the developing brain have not been fully elucidated, abundant evidence shows that apoptosis is one of the factors involved both in vivo and in vitro (Cheema et al., 2000; Ikonomidou et al., 2000; Ramachandran et al., 2003; Smith et al., 2015). EtOH may have different effects on immature and mature neurons, acting as a stimulant in the former and as a depressant of

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; GFAP, gliofibrillary acidic protein; MAP2, microtubule-associated proteins 2; EtOH, ethanol; TUNEL, dUTP-mediated nicked end labeling; CNS, Central Nervous System; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay; AO, acridine orange; EB, ethidium bromide; PBS, phosphate buffered saline; SSC, saline sodium citrate buffer; MT, microtubules; Caspase 9a, Caspase 9 active; Caspase 3a, Caspase 3 active; Syn, Synaptophysin; PSD95, Postsynaptic density protein 95.

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the central nervous system (CNS) in the latter (Galindo et al., 2005). Some studies have shown that neurons develop resistance to the effects of EtOH as they mature (Mooney and Miller, 2007; Mameli et al., 2005). Further reports show that the administration of doses of EtOH that might cause extensive neuronal loss in neonatal mice are not associated to neuronal loss in the adult brain, a difference which may also depend on the animal's genetic profile (Linsenbardt et al., 2009; Itzhak and Anderson, 2008).

The consequences of EtOH exposure during pregnancy are partly due to a direct cytotoxic effect on immature neurons, which are particularly sensitive to EtOH during the synaptogenetic period, characterized by neurite elongation, synaptic contact formation and the onset of interneuronal signalling (Kyzar and Pandey, 2015). Normal cognitive function depends on adequate neuronal differentiation and migration, proper axonal arborization and the correct formation of synapses at contact sites, mainly on dendritic spines. Although the effects of EtOH exposure on the neuronal cytoskeleton during differentiation need further elucidation, some reports indicate they might constitute an underlying alcohol toxicity mechanism in the brain (Ahluwalia et al., 2000; Depaz et al., 2005; Sordella and Van Aelst, 2006; Evrard and Brusco, 2011). Other studies have focused on the effect of EtOH on alterations in membrane neuronal traffic to the dendritic profiles, which are involved in neuron functionality (Romero et al., 2015).

We have previously shown that the cerebral cortex is particularly susceptible to the effects of prenatal EtOH exposure (Aronne et al., 2011). EtOH induces a reduction in cerebral cortex total mass and thickness, together with a decrease in the number of neurons (Aronne et al., 2011). Various studies in rats and humans further suggest that chronic exposure to EtOH during early development leads to massive cortex disorganization characterized by heterotopic neuron clusters and astrogliosis (Miller and Robertson, 1993).

The cellular processes underlying the disruption in dendrite growth and synapse formation are difficult to elucidate in animal models of brain development, as they are rarely used to study individual neurons. In this context, the current study sought to overcome these limitations by using low-density cultures of postnatal cortical neurons in which individual neurons could be subjected to quantitative analysis of dendritic arborization. Therefore, and in order to analyze the direct effect of EtOH on cortical neurons during the period of differentiation, primary cultures of cortical neurons obtained from 1-day-old rats were exposed to EtOH after 7 days of culture, and viability and morphology were analyzed at structural and ultrastructural levels.

2. Results

2.1. EtOH decreased cortical neuron viability

Neuronal viability was determined by the MTT assay after 24 h EtOH exposure. As shown in Fig. 1, EtOH induced a significant reduction in the viability of cultured cortical neurons. Fifty mM and 100 mM EtOH induced a decrease of 73 ± 7% and 65 ± 9% in cell viability, respectively. The lowest concentration of EtOH assayed (25 mM) did not modify cell viability.

2.2. EtOH induced apoptosis in rat cerebral cortex neurons

The type of cell death induced by EtOH was determined through morphological analyses and nuclear morphology was evaluated by differential nuclear staining with fluorescent dyes AO and EB. AO permeates all cells and stains nuclei green, while EB is only taken up by cells when cytoplasmic membrane integrity is lost and stains nuclei red. Therefore, live cells have a normal green nucleus, early apoptotic cells have a bright green nucleus with condensed or

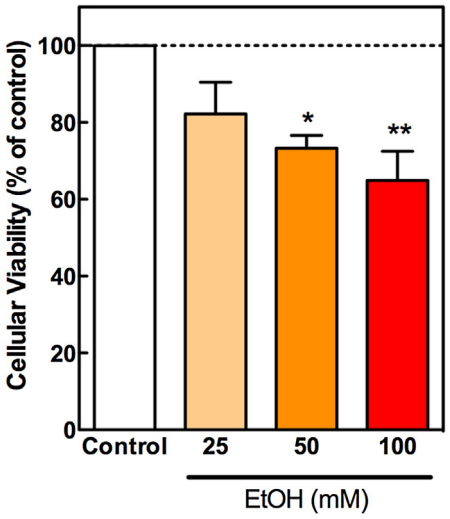

Fig. 1. Percentage of viable neuronal cells measured by the MTT method. Results were expressed as mean ± SEM of four independent experiments performed in sextuplicate. *p<0.05 vs. control (one-way ANOVA followed by Bonferroniís post hoc test).

fragmented chromatin, late apoptotic cells display condensed and fragmented orange chromatin and necrotic cells exhibit a structurally normal orange nucleus (Fig. 2A).

The amounts of viable, necrotic and apoptotic cells resembled control values (data not shown) after 12-h incubation with 25 mM, 50 mM or 100 mM EtOH and after 24-h incubation with 25 mM EtOH. In contrast, 24-h incubation with 50 mM and 100 mM EtOH induced a significant decrease in the number of nuclei compatible with viable cells (Fig. 2B). Only 100 mM EtOH treatment for 24 h induced an increase in the number of nuclei compatible with necrotic cells (Fig. 2C).

The percentage of apoptotic cells increased with 50 and 100 mM EtOH treatment (Fig. 2D), while no differences were found between 25 mM EtOH treatment and the control group. These data suggest that 50 to 100 mM EtOH treatment preferentially induces apoptotic death in primary cultures of cortical neurons.

To further corroborate the pro-apoptotic effect of EtOH, we evaluated DNA damage by TUNEL staining in cortical neurons incubated in the absence or presence of EtOH. As shown in Fig. 3A, nuclei from untreated cells (control) appeared with typical morphological features of neuronal nuclei (blue stain with Hoechst). However, after 24-h exposure to 50 mM and 100 mM EtOH, an increase was observed in DNA nicks localized in the nuclei (green stain with TUNEL). After 50 and 100 mM EtOH treatment during 24 h, 35–45% of cortical neurons were TUNEL positive (Fig. 3B).

We further analyzed caspase 3a and caspase 9a expression in cortical neurons exposed to EtOH. They are key regulators involved in cellular apoptosis. As shown in Fig. 3C, 100 mM EtOH induced an increase in caspase 3a and caspase 9a expression.

2.3. EtOH reduced the number of MAP-2-positive cells and induced morphological changes in dendritic profiles

After 24-h exposure to 50 mM and 100 mM EtOH, primary cultures exhibited a decrease in the number of MAP-2-positive neurons, with values around 74 ± 7% and 57 ± 8%, respectively, compared to control (data not shown). In addition, neuronal morphology displayed alterations, showing fewer prolongations (Fig. 4A).

Concerning length, 50 and 100 mM EtOH treatment induced a significant reduction in total dendrite length (Fig. 4B), which is a

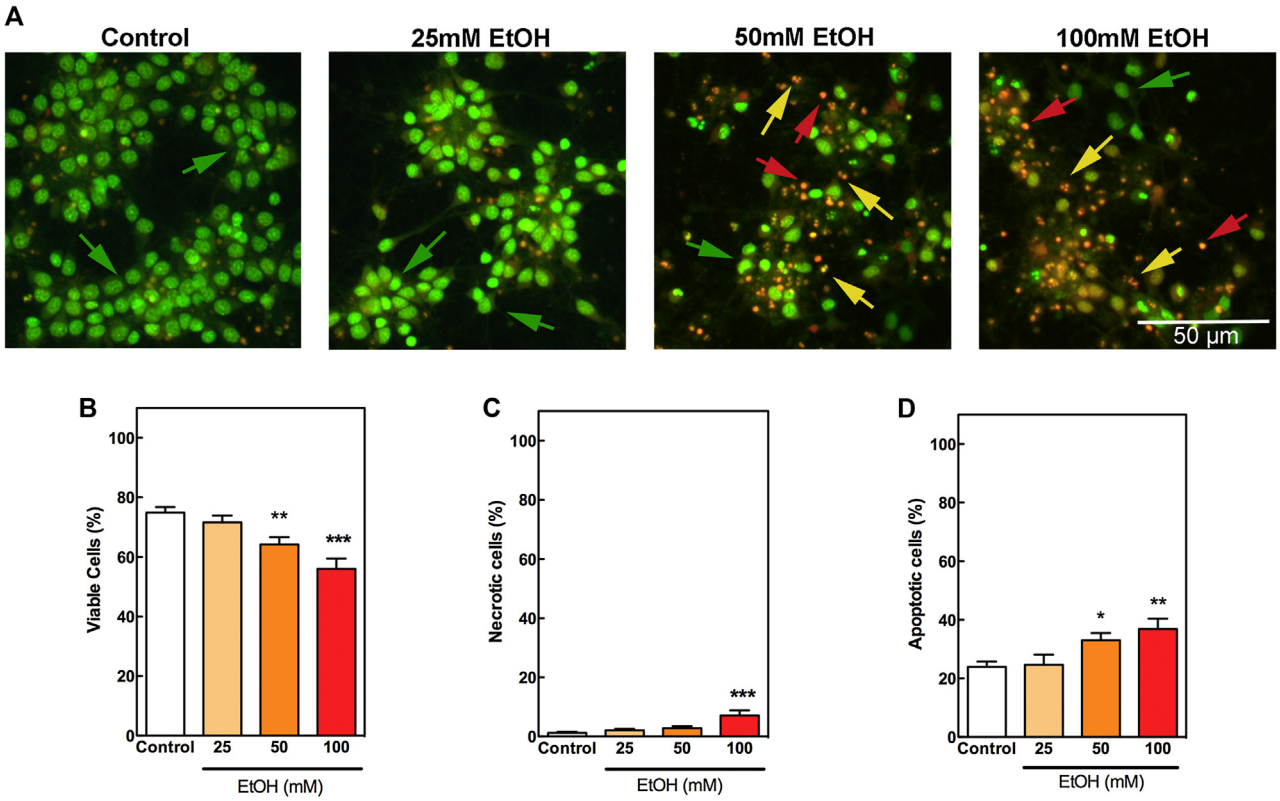

Fig. 2. EtOH induced an increase in neuronal cellular apoptosis. Primary neuronal cortical cells were incubated in the absence or presence of EtOH and viable, apoptotic or necrotic cells were evaluated as described under Methods. (A) Representative images of viable (green arrow), necrotic (red arrow) and apoptotic (yellow arrow) neuronal cells. Percentage of viable (B), necrotic (C) and apoptotic (D) neuronal cells measured by AO-EB staining. Results were expressed as mean ± SEM of three independent experiments (10 microscopic fields (10×) analyzed per sample). Scale bar: 50 µm for all images. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA followed by Bonferroniís post *hoc* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

function of individual dendrite length and dendrite number. EtOH treatment did not affect the primary dendrite number per cell (Fig. 4C). In addition, exposure to 50 mM for 24 h decreased primary dendrite length in individual cells (Fig. 4D), while 100 mM EtOH for 24 h reduced both primary dendrite length and secondary dendrite number per cell (Fig. 4E). 25 mM EtOH had no effects (Fig. 4F).

2.4. EtOH induced ultrastructural changes in primary cultures of cortical neurons

Morphological changes induced by EtOH in cortical neurons were investigated at ultrastructural level. Fifty mM EtOH exposure caused nucleus indentation (Fig. 5B), increased the number of lipidic vacuoles, lysosomes and multilamellar vesicles and induced a dilated endoplasmatic reticulum lumen and a disorganized Golgi apparatus with a ring-shape appearance (Fig. 5D–H). Furthermore, EtOH treatment induced a disorganized distribution of MT, which were also decreased in the neuronal processes (Fig. 5J,K).

The growth cones from EtOH-exposed neuronal cultures showed cytoskeletal disorganization regarding controls (Fig. 6B–D). Regarding neuronal synapses, normal presynaptic vesicle distribution was observed in control conditions, while EtOH treatment induced apposition between pre- and postsynaptic membranes without a defined synaptic cleft and showing a delay in presynaptic vesicle organization (Fig. 6B).

Specific proteins present in synapses like Syn and PSD95, pre- and postsynaptically located, were more lowly expressed in synapses of 100 mM EtOH-exposed cultures (Fig. 6E,F) and correspond to immature synapses.

3. Discussion

Our study shows that EtOH induced apoptosis in neuronal cultures from cerebral cortex of 1-day-old rats. EtOH-induced apoptosis was accompanied by a decrease in dendrite arborization and ultrastructural alterations in nuclei and cytoplasmatic organelles, as well as in cytoskeletal organization.

EtOH has been previously shown to produce neuronal cell damage during prenatal development and fetal alcohol syndrome. EtOH has apoptogenic properties which damage both CNS progenitors and partially and fully differentiated brain cells, depending on the developmental age at which alcohol exposure takes place (Olney, 2014). This could explain the neuropsychiatric disturbances associated with fetal alcohol syndrome (Farber et al., 2010).

The type of cell death –apoptosis or necrosis– changes with the timing and extent of the insult (Nicotera et al., 1999). In our study, exposure to EtOH resulted in a reduction in cortical neuron viability and, although it is difficult to directly correlate EtOH concentration in vitro with blood EtOH levels in vivo, the neuronal apoptosis that EtOH induces in vivo appears to be faithfully reproduced in the in vitro experimental system.

In this study, exposure to EtOH during 24 h after 7 days of development of cortical neurons in vitro resulted in a decreased dendritic arbor size. This decrease is accounted for by two factors: fewer dendrites per cell and shorter individual dendrites. These findings are consistent with previous reports of inhibitory effects of EtOH on dendritic arbor size in hippocampal neurons (Davies and Smith, 1981). Perinatal EtOH exposure severely compromises the dendritic arbor and produces changes in the organization of the neurophil, as well as alterations in the organization of the perikaryal organelles (Smith and Davies, 1990). Yanni and Lindsley (2000)

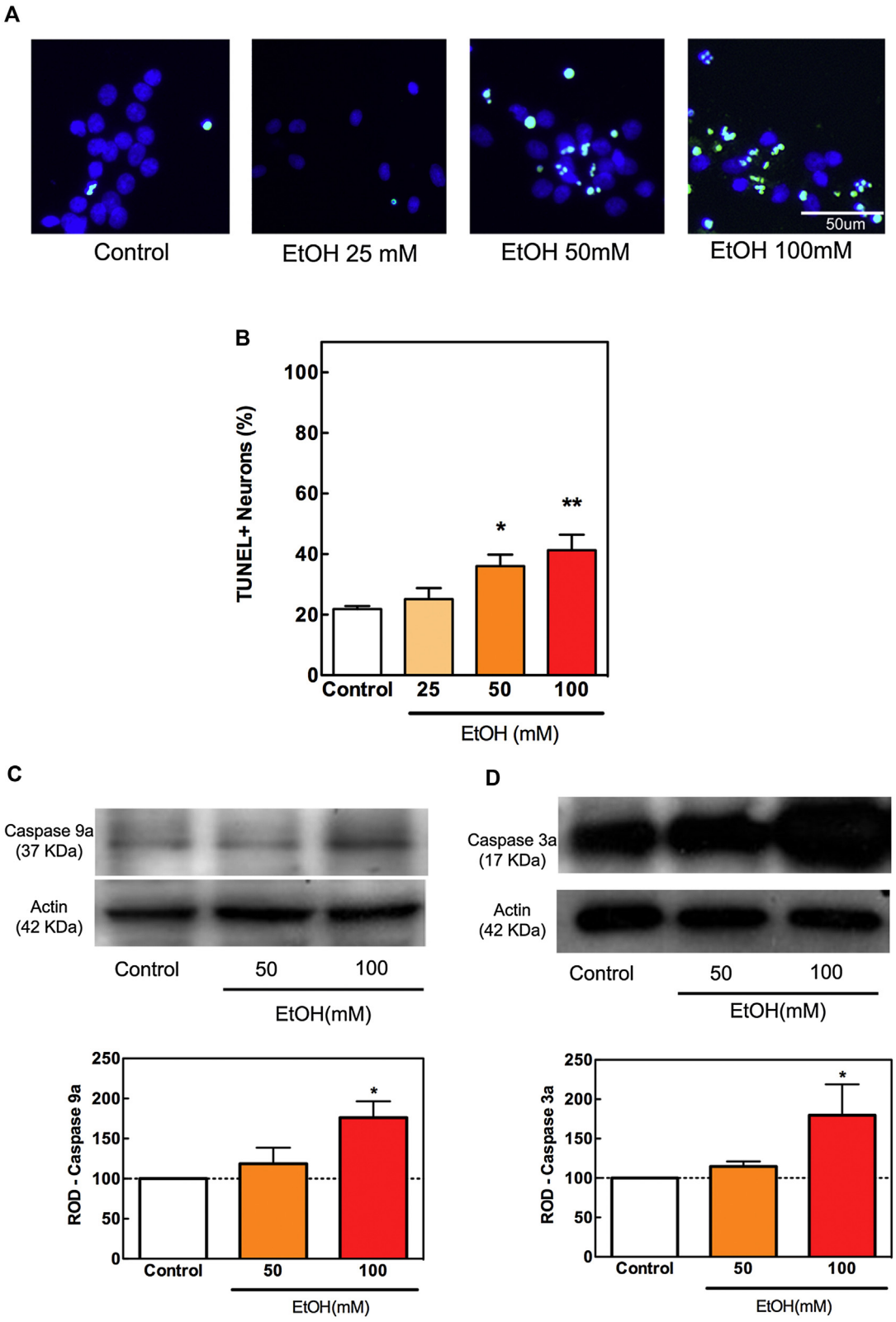

Fig. 3. Ethanol induced neuronal death. Primary neuronal cortical cells were incubated in the absence or presence of EtOH and neuronal death was evaluated by TUNEL assay as described under Methods. (A) Representative images of neuronal cells subjected to Hoechst 33342 (blue) and TUNEL (green) staining. Scale bar: 50 μm for all images. (B) Percentage of TUNEL positive neurons. Results were expressed as mean ± SEM of three independent experiments (10 microscopic fields (20×) analyzed per sample). *$^{*}p < 0.05$, $^{**}p < 0.01$* (one-way ANOVA followed by Bonferroniís post *hoc* test). (C) Effect of EtOH on caspase 9a and (D) caspase 3a expression in neuronal cells incubated in the absence (control) or presence of EtOH during 24 h. Results were expressed as fold change of the response detected in control conditions. Each bar represents the mean ± SEM of 4 independent preparations. * P < 0.05 vs. control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

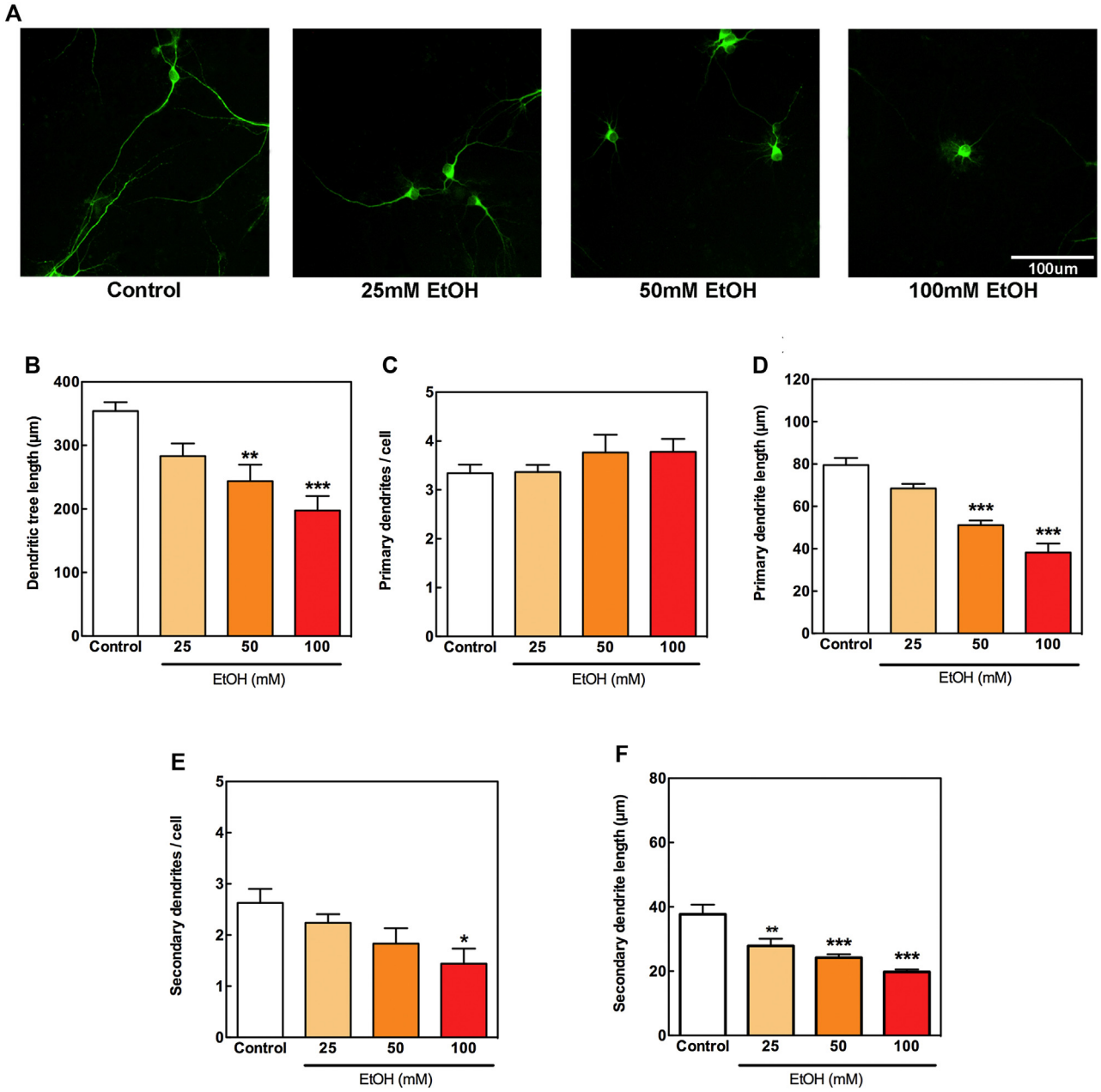

Fig. 4. Ethanol reduced the number of MAP-2 positive cells and induced morphological changes. (A) Representative images of MAP2 immunostaining in cortical neuronal cells incubated in the absence (control) or presence of EtOH during 24 h. Scale bar: 100 μm for all images. Total dendritic length (B), number of primary dendrites per cell (C), primary dendritic length (D), number of secondary dendrites per cell (E) and secondary dendritic length (F) were evaluated. Results were expressed as the mean ± SEM of three independent experiments (10 microscopic fields (10×) analyzed per sample). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (one-way ANOVA followed by Bonferroni's post hoc test).

have reported that six-day treatment of rat hippocampal pyramidal neuron cultures with EtOH (200, 400 or 600 mg/dl) resulted in a decrease in total dendritic length per cell, dendrite number per cell, length of individual dendrites and synapse number per innervated dendrite, but had no effects on cell survival, probably due to the probably protective properties of B-27, the culture medium employed.

In turn, the study of cytoskeletal proteins in neuronal cultures from different brain areas has shown actin filaments to be very sensitive. F-actin decreases in cerebellar cells in vitro as a consequence of EtOH exposure (Popp and Dertien, 2008). Moreover, a reduction in F-actin is observed in hippocampal neurons at 14 days in vitro in the presence of EtOH, without changes in total actin (Romero et al., 2010 and Romero et al., 2013). Our findings in cortical neurons in vitro show no changes in total actin but a reduction in dendritic arborization and synaptic contacts, which could be speculated to reflect the alterations in F-actin observed in the above mentioned manuscripts.

Recently, results by Romero and collaborators (2015) showed that EtOH exposure produces disorganization of endoplasmatic reticulum and Golgi Apparatus with dilated intracisternal lumen. These data correlate with the phosphorylation of cytoskeleton proteins, principally actin, and changes in calcium availability needed for neurons functionality (Romero et al., 2015). In agreement with these results, our present study shows that EtOH induces endomembrane alterations in primary neuronal cultures from cerebral cortex, with dilated lumen being more evident in endoplasmatic reticulum and Golgi Apparatus.

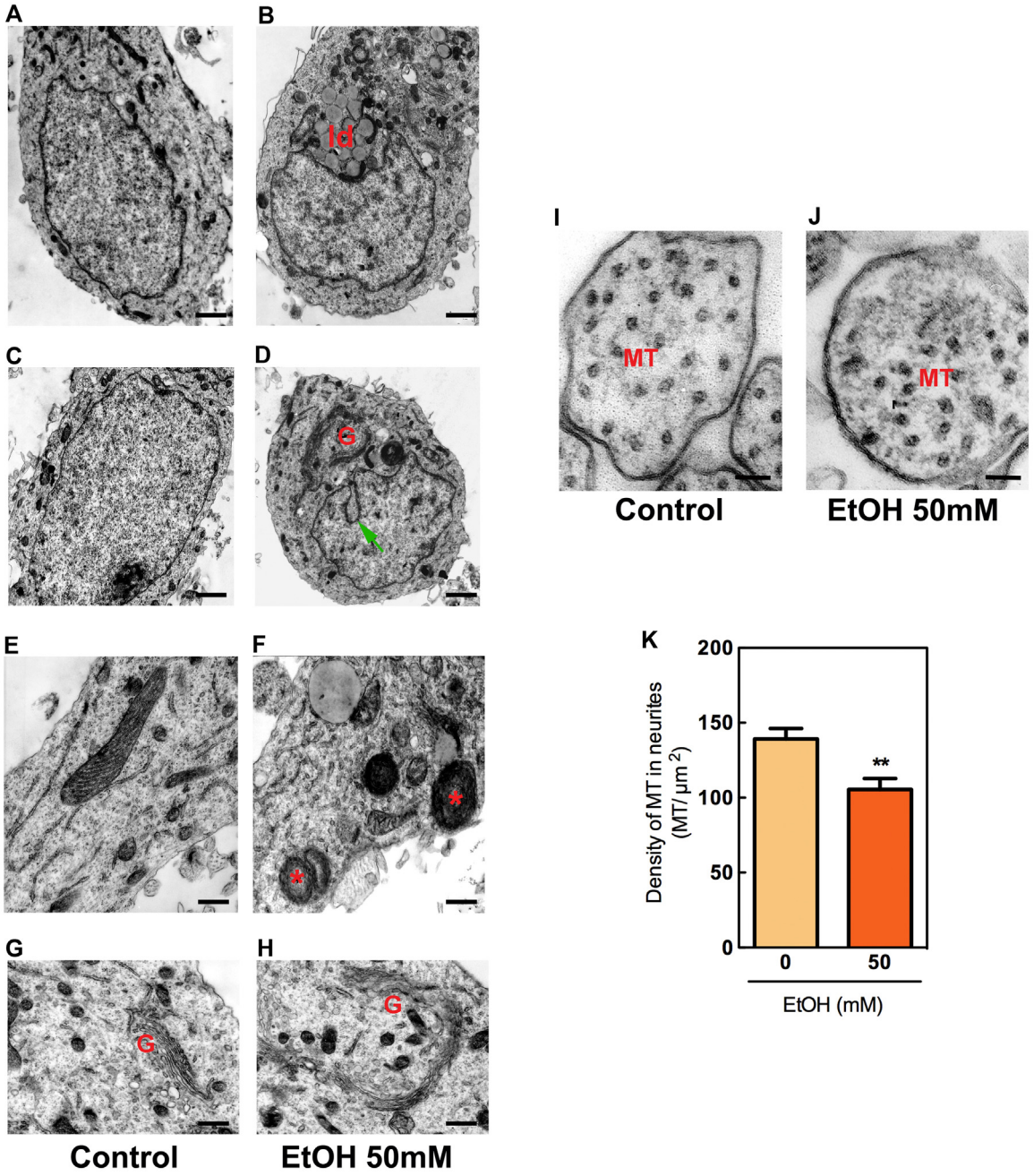

Fig. 5. Ethanol-induced cytoskeleton and endomembrane alterations in cortical neurons. Primary cortical neuronal cells were incubated in the absence (A, C, E, G and I) or presence of 50 mM EtOH (B, D, F, H, and J) for 24 h and ultrastructural changes were analyzed by electron microscopy. Images of EtOH-treated neurons show nuclear shrinkage, nuclear indentation (green arrow), ring-shaped Golgi apparatus (G), multilamellar organelles (red asterisks), lipid droplets (Ld) and asymmetric distribution of microtubules (MT) along the neuronal processes. The number of microtubules per area (MT/μm^2) was evaluated in transversal sections of neuronal processes (10 photographs of each one of three different experiments) and expressed as density of MT in neurites. ** $P < 0.01$ vs. control. Scale bars: 100 nm (A,B,C, D); 50 nm (E,F,G,H) and 100 nm (I,J). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Apoptotic neurons present in our cultures exposed to EtOH are shown in Figs. 2 and 3. Since caspase-mediated degradation of the cytoskeleton is directly involved in mediating axon blebbing and fragmentation, as proposed by Sokolowski et al. (2014), caspase activity cannot be ruled out as the cause for the disorganization of the cytoskeleton observed in neurons exposed to EtOH (present results). In fact, we show that caspase 3 and 9 were increased by EtOH treatment (Sokolowski et al., 2014).

It has been shown that EtOH-induced cellular apoptosis on primary developing hypothalamic neurons is mediated by an increase in neuronal oxidative stress and the production of microglia-derived factors (Boyadjieva and Sarkar, 2013). In our studies, the involvement of factors released by microglial cells can be easily ruled out, as our culture medium was solely for neurons and glial cells die in this medium. And, although we used a similar time of differentiation of primary neuronal cultures, the same time of exposure and the same concentration of EtOH, our cultures lacked microglial cells or molecular factors derived from them. Therefore, we may speculate that the effect of EtOH on the apoptotic process may be due to a direct effect of this neurotoxic agent on differentiating neurons.

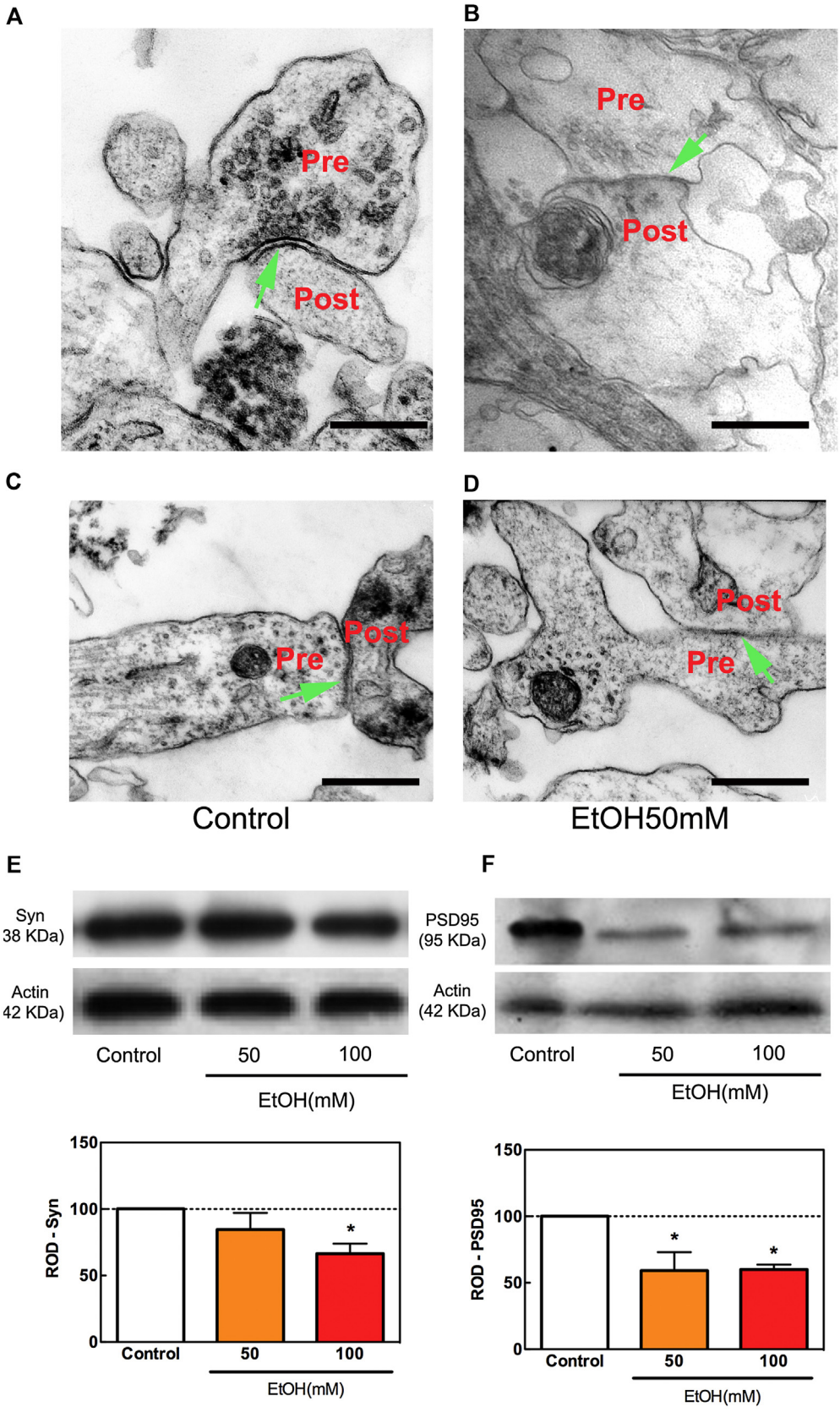

Fig. 6. Ethanol induced ultrastructural changes in the neuronal synaptic contacts. Primary neuronal cortical cells were incubated in the absence (A and C) or presence of 50 mM EtOH (B and D) for 24 h and synaptic contacts were analyzed by electron microscopy. (E) Effect of EtOH on Syn and (F) PSD95 expression in neuronal cells incubated in the absence (control) or presence of EtOH for 24 h. Results were expressed as fold change of the response detected in control conditions. Each bar represents the mean ± SEM of 4 independent preparations. * $P < 0.05$ vs. control. Scale bars: 500 nm.

Taking into account that cells underwent apoptosis under EtOH treatment (Fig. 3) and that this agent induced the appearance of multilamellar vesicles as well as changes in mitochondria and reticulum, cells may be thought to respond to EtOH through autophagic processes in order to reduce damage. In agreement, Chen and collaborators (2012) have demonstrated both in vivo (cerebral cortex of 7 days old mice) and in vitro (neuroblastoma) that autophagy is the mechanism through which EtOH-induced apoptosis is modulated (Chen et al., 2012).

Naseer et al. (2014) demonstrated similar results in primary cultures of hippocampal neurons and suggested drugs such as osmotin and metformin as neuroprotectors or preventers of the apoptotic process induced by EtOH by the activation of adenosine monophosphate-activated protein kinase (Naseer et al., 2014).

EtOH-induced synaptic alterations may be correlated with the amount of PSD95 present in the postsynaptic contact zone. Kyzar and Pandey (2015) have examined dendritic remodeling during brain EtOH exposure in vivo, concluding that dendritic remodeling depends on the activity of regulatory molecules of cytoskeletal proteins in actin filaments present in the dendritic spines. Alterations in synaptic profiles are related with the PSD95 expression. In this way, we show that cortical neuron cultures exposed to EtOH resulted in a decreased expression of PSD95 and immature neuronal contacts (see Fig. 6), probably due to the direct effect of EtOH on cytoskeletal protein organization, which in turn alters the structure of synaptic contact.

In conclusion, our study shows that EtOH induces neuronal apoptosis and changes in the cytoskeleton and membrane proteins related with the establishment of mature synapses. Altogether, these novel findings on cell structural and ultrastructural changes provide pioneering evidence on the direct effects of EtOH on neurons, which may partially explain its effects on brain development.

4. Experimental procedure

4.1. Drugs and reagents

Neurobasal medium, B27 and Dulbecco's modified Eagle's medium (DMEM) were from Invitrogen (Carlsbad, CA, USA). Dnase I, poly-L-lysine, Hoechst 33342, acridine orange, ethidium bromide, osmium tetroxide, Epoxy Embedding Medium Kit, polyclonal rabbit anti-gliofibrillary acidic protein (GFAP) and the monoclonal mouse anti-brain microtubule-associated proteins 2 (MAP2) were purchased from Sigma-Aldrich (St Louis, MO, USA). Papain was from Worthington (Lakewood, NJ, USA). EtOH was from Merck (Kenilworth, NJ, USA). dUTP-mediated nicked end labeling (TUNEL) assay kit was purchased from Promega (Madison, WI, USA). The rest of reagents were of analytical grade.

4.2. Experimental animals

One-day-old Wistar rats born to nuliparous pregnant females were used. Pregnant rats were housed in the animal lab at the Institute of Cell Biology and Neuroscience, following current protocols for animal care and minimizing the number of litters used. Animal care for this experimental protocol was also in accordance with the NIH guidelines for the Care and Use of Laboratory Animals, the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and with the CICUAL Animal Protocol at of the School of Medicine (University of Buenos Aires), Res CD 2209/2015.

4.3. Cell culture

Neuronal cultures were prepared as previously described by Lopez Verrilli et al. (2009). Briefly, the cerebral cortices of 1-day-old Wistar rats were dissociated with 18 U/ml papain at 37 °C for 30 min. After 5-min centrifugation at 600 × *g*, the resulting pellet was triturated in D-MEM containing Dnase I (0.01 mg/ml) and centrifuged at 800 xg for 5 min. Cells were resuspended in neurobasal medium supplemented with 2% B-27, 1% glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and plated in poly-L-lysine-pre-coated tissue culture dishes. Cortical neurons were allowed to complete differentiation in control media for a total of 5-6 days. Neuronal cells were characterized by double immunofluorescence for MAP2 (a neuronal marker) and GFAP (an astroglial marker), which revealed nearly 95% of the cellular population corresponding to neurons (data not shown). Cellular experimental treatment was carried out in 16-h B27-starved conditions. EtOH concentration in the media was maintained at ≥85% from the initial concentration using a chamber system previously described by Eriksen et al. (2002).

4.4. Cell viability

Cell viability was determined using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay based on the cleavage of the yellow tetrazolium salt –MTT– to purple formazan by mitochondrial enzymes in metabolically active cells (Denizot and Lang, 1986). Neuronal cells (100,000 cells/well in 96-well plate) were incubated in the absence or presence of EtOH (25, 50, 100 mM) for 24 h. MTT was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h at 37 °C. The dye was solubilized with 0.04 N HCl in isopropanol and the absorbance of each sample was measured at 595 nm in a 96-well plate reader (Biotrak II Reader). Quantification was done on the basis on 4 independent experiments, each of them repeated six times. Results were expressed as a percentage of control (100% in untreated cells).

4.5. Nuclear morphology

Nuclear morphology was analyzed using the fluorescent probes acridine orange (AO) and ethidium bromide (EB) (AO-EB staining), which differentially stain cell nuclei and viable (green), apoptotic (orange) and necrotic (red) cells (Baskic et al., 2006). Primary cultures of cortical neurons were incubated in the absence or presence of 25, 50,100 mM EtOH during 24 h. Culture media were then replaced and a solution containing the probes AO and EB (0.1 mg/ml each) in phosphate buffered saline (PBS) was added. Cells were further incubated for 5 min at room temperature to allow probe incorporation and washed in warm PBS. Cellular nuclear morphology was immediately observed through a Zeiss Axiolab microscope with a coupled digital camera (Olympus Optical Co., Ltd, Japan). Viable, apoptotic and necrotic cells were then quantified using Image Pro Plus 5.1 software (Media Cybernetics Inc., Bethesda, MD, USA).

4.6. DNA fragmentation

Detection of DNA double-strand breaks by TUNEL was performed according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, cortical neurons were incubated in the absence or presence of 25, 50,100 mM EtOH during 24 h. Cells were then fixed for 20 min in warm 4% paraformaldehyde in PBS and later incubated in equilibrium buffer for 10 min, followed by reaction buffer (equilibrium buffer, nucleotide mix with fluorescein conjugated dUTPs and terminal deoxynucleotidyl transferase) for 60 min. The reaction was stopped with saline sodium citrate buffer (SSC) 2× for 15 min, washed in PBS and counter-stained with Hoechst 33342 (2 μg/ml Sigma-Aldrich) to label nuclei and coverslipped with anti-fading mounting medium Fluoroshield (Sigma-Aldrich).

Images were obtained using an Olympus Q color 3 digital camera added to a fluorescence Zeiss Axiolab microscope.

4.7. Immunofluorescence staining

Cortical neuronal cells were fixed for 20 min in warm 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After blockade with 5% normal goat serum for 30 min, cells were incubated with mouse monoclonal anti-MAP-2 antibody, a marker for neuronal soma and dendrites (1:1000), at 4 °C overnight. Cells were then incubated with anti-mouse IgG FITC conjugate (1:500) for 4 h at room temperature, later washed in PBS and finally counterstained with 2 μg/ml Hoechst 33342 for 10 min. Images were obtained using a fluorescence Zeiss Axiolab microscope with an Olympus Q color 3 digital camera. For negative controls, cells were treated with normal goat serum in the absence of the primary antibody and no stain was visible (data not shown).

4.8. Cellular morphology

Cells were fixed and immunostained for MAP-2 as described above. Immunoreactive cells were examined in different randomly selected microscopic fields on a minimum of three dishes for each experiment, and at least three experiments for each condition. Only isolated neurons whose cell bodies or processes were not in contact with other neurons were analyzed. Images were taken with an Olympus Digital Camera coupled to a Zeiss Axiolab microscope using a 10× objective, which was sufficient to visualize the entire dendritic arborization.

The number of primary and secondary dendrites per neuron, primary and secondary dendrite length (μm) and total dendrite length per cell were analyzed in MAP-2-positive cells through Image Pro Plus software. The total length of dendritic arborization per neuron was determined by calculating the sum of the neuronís individual dendrite lengths.

4.9. Electron microscopy

Samples of three different experiments were used for ultrastructural studies. The plastic culture dishes containing neurons were washed in PBS. Cells were fixed “in flat” with 1.5% glutaraldehyde + 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4 °C for 60 min and later postfixed in 2% osmium tetroxide containing 0.8% potassium ferrocyanide in cacodylate buffer for 60 min. Neurons were then treated with 0.1% tannic acid in the same 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. Cells were then dehydrated in EtOH and finally embedded as monolayers in Epoxy Embedding Medium Kit according to modifications of the Abd-el Basset et al. (1992) procedure. Briefly, cell monolayers were covered with a layer of 2–3 mm of epoxy resin dissolved in absolute EtOH (1:1) until epoxy-embedding medium alone was left, and later kept at 60 °C for 3 days until polymerization was complete. The cell monolayers were removed from the dishes immediately and clamped into a resin capsule to obtain ultrathin sections (Deitch and Banker, 1993). Sections were counterstained with acetate uranyl (20 min) and lead citrate (1 min) and then examined and photographed in a Zeiss EM 109 electron microscope at 12000× and 50000× primary magnification. Microtubules (MT) were quantified in randomly selected cross-sectioned processes of both control and EtOH-exposed neurons (n = 20 processes, one per neuron) and recorded at 30000×. Micrographs were analyzed with ImageJ software and the area of each profile was determined. The numerical density of MT is the number of MT present in a pre-defined area (μm2).

4.10. Western blot

Samples were processed and subjected to Western blot as previously reported (Lopez Verrilli et al., 2009). The antibodies used for immunoblot were: rabbit anti-Caspase9 active (Caspase 9a, Cell Signaling; 1:200), rabbit anti-Caspase 3 active (Caspase 3a, Cell Signaling; 1:200), mouse anti-Synaptophysin (Syn, Sigma-Aldrich;1:500), mouse anti-postsynaptic density protein 95 (PSD95, Sigma-Aldrich; 1:1000) and rabbit anti-actin (1:1000). Actin expression was tested in control and EtOH-exposed neurons and, as no changes were observed, actin was used as loading control.

Optical densities of the relevant immunoreactive bands were quantified by ImageJ (http://imagej.nih.gov).

4.11. Statistical analysis

Results were expressed as the mean ± SEM. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni post-tests (GraphPad Prism 4, GraphPad Software Inc., La Jolla, CA, USA) or Student's *t* test when appropriate. P values <0.05 were considered significant.

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Conflict of interest

All authors declare no conflict of interest.

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