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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

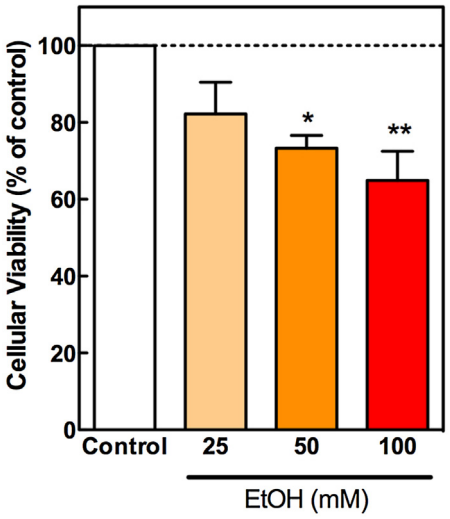

Fig. 1. Percentage of viable neuronal cells measured by the MTT method. Results were expressed as mean ± SEM of four independent experiments performed in sextuplicate. *p<0.05 vs. control (one-way ANOVA followed by Bonferroniís post hoc test).

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