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Ethanol-Induced Changes in Neuron and Astrocyte number in the Rat Cerebral Cortex

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Abstract.- Chronic consumption of ethanol can alter brain morphology and function. The present study examined the effect of ethanol abuse on the number of neurons and astrocytes in the rat cerebral cortex. After 45 days of ethanol treatment (2 g/kg by intraperitoneal injection), tissue samples from the rat motor cortex were Nissl-stained and probed for glial fibrillary acidic protein (GFAP) expression. The number of neurons and GFAP-immunoreactive astrocytes in each cortical layer were counted per unit area. The results showed that ethanol-treated rats had fewer neurons ($P < 0.01$) but a greater number of GFAP-positive astrocytes in all cortical layers as compared to control-treated animals ($P < 0.01$). Neuronal loss was the most pronounced in layers III and V, the major output layers of the cortex, corresponding to increased astrocyte density in these layers. These results provide evidence that chronic ethanol exposure induces neuronal death but stimulates astrocyte hyperplasia in the cerebral cortex, especially in the output layers. Loss of neurons may lead to damage to neural circuitry, while astrocyte proliferation likely serves a protective function to compensate for the resultant dysfunction of the nervous system.

Keywords: Ethanol-induced changes, cerebral cortex, neuron, astrocyte, Sprague-Dawley rat.

INTRODUCTION

Ethanol is a neurotoxic substance, and its abuse has adverse effects such as motor impairment, cognitive disorders, and psychopathy (Hanchar *et al*., 2005; Sabia *et al*., 2014). Ethanol induced changes in the brain are well documented, and include a reduction of brain volume (Beck *et al*., 2012), neuronal apoptosis (Ramachandran *et al*., 2003), a decline in synaptic response (Crowley *et al*., 2014; Zorumski *et al*., 2014), organelle degeneration (Jaatinen and Rintala, 2008; Reddy *et al*., 2013), neurotransmitter imbalance (Tiwari *et al*., 2014), alterations in neuronal firing (Kozhechkin *et al*., 2013; Botta *et al*., 2014) and behavioral retrogression (Sabia *et al*., 2014), all of which are correlated with neurological dysfunction. On the other hand, the nervous system also has a mechanism to compensate for ethanol-induced neurotoxicity; for example, chronic ethanol exposure stimulates GFAP synthesis (Dalcik *et al*., 2009; Udomuksorn *et al*., 2011), and enhances glial activity (Miguel-Hidalgo *et al*., 2006; Kane *et al*., 2014), as well as increases expression of S100 protein (Shen *et al*., 2014), which may contribute to the maintenance of neural homeostasis. However, the precise changes that occur in neurons and astrocytes in each cortical layer upon ethanol toxicity are unknown. In this study, a quantitative analysis of neuronal and astrocyte density in each cortical layer was carried out in rats receiving chronic ethanol treatment. The results indicate that neuronal loss is accompanied by an increase in astrocyte number in all layers and is especially evident in the output layers of the cerebral cortex as a result of ethanol-induced toxicity.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats ($n = 12$; 2 months old, 230–250 g) were randomly assigned to one of two groups ($n = 6$ in each): the experimental group received ethanol administration, 2 g/kg body weight dissolved in 1 ml saline, once per day for 45 consecutive days injected intraperitoneally,

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according to a well-established protocol (Yoshimoto and Komura, 1993; Shen *et al*., 2014), while the control group received only vehicle treatment (1 ml of 0.9% NaCl). Rats were monitored daily for symptoms of toxicity and weighed weekly to assess general health status. Animals were individually housed in a temperature-controlled (22±2°C) room under a 12:12 h light/dark cycle with free access to food and water. Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue preparation

Rats were anesthetized with sodium pentobarbital (40 mg/kg by intraperitoneal injection) and then perfused with 100 ml of 0.9% NaCl through the left ventricle, followed by 100 ml of fixative solution containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). the left primary motor cortex was dissected according to rat brain atlas coordinates (Swanson, 1998). After additional fixation overnight in the same solution, tissue blocks were trimmed and washed in PBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Serial 6-μm-thick coronal sections were cut and mounted on microscope slides coated with 3-aminopropyl triethoxysilane (Sigma–Aldrich, St. Louis, MO, USA; 1:50 in acetone) for histological staining.

Nissl and immunohistochemical staining

Three sections 180 μm apart were selected from each brain for Nissl staining. Sections were deparaffinized in xylene, rehydrated in a graded series of ethanol and water, and then incubated in 0.5% thionine for 40 min at 37°C. After dehydration in a graded series of ethanol and clearance in xylene, slides were mounted in Permount.

Adjacent sections were processed for immunohistochemistry according to a previously published protocol (Shen *et al*., 2014). Briefly, sections were sequentially incubated with 3% H_2O_2 for 5 min, 5% goat serum (Vector Laboratories, Burlingame, CA, USA) for 10 min, a rabbit monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:400; Sigma–Aldrich) for 10 min, biotinylated anti-rabbit IgG (1:100; Sigma–Aldrich) for 10 min, and a preformed avidin–biotin–peroxidase complex (Sigma–Aldrich) for 10 min. All incubations were at 37°C. Sections were then incubated in 0.05% 3,3-diaminobenzidine/0.01% hydrogen peroxidase (Sigma–Aldrich) in PBS for 10 min at 37 °C to visualize GFAP immunoreactivity. Negative controls were processed in parallel by substituting PBS for the primary antibody.

Quantitative analysis

Cell number was quantified by microscopy in three randomly chosen visual fields according to previously described methods (Shen *et al*., 2014) at 400× magnification in each cortical layer on each slide using a calibrator (125 × 125 μm). Cells were counted by an investigator blinded to the treatment condition.

The criteria for identifying a neuron in Nissl-stained sections were a clear differentiation of the soma from background staining and a nucleus. Astrocytes were identified as GFAP-positive cells with a labeled soma and several radial processes.

Statistical analysis

All data are presented as mean ± standard error of the mean. Statistical significance was evaluated by the Student's *t* test or one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

RESULTS

After 45 days of ethanol treatment, rats showed obvious symptoms of alcoholism, such as dyskinesia, ataxia, and a delayed response to various stimuli, consistent with previous observations of behavioral impairment due to ethanol-induced toxicity in rodents (Yoshimoto and Komura, 1993; Shen *et al*., 2014). The body weight in ethanol-treated rats was slightly lower than that of control animals, but the difference was not statistically significant (data not shown).

Ethanol-induced changes in neuronal density in the cerebral cortex

The cerebral cortex is histologically divided into the following six layers from the pial surface to the white matter according to characteristic neuronal

distribution: molecular (layer I), outer granular (layer II), outer pyramidal (layer III), inner granular (layer IV), inner pyramidal (layer V), and multiform (layer VI) (Fig. 1A). The distribution of neurons (Fig. 1A and 1B) and GFAP-expressing astrocytes (Fig. 1C and 1D) can be clearly discerned in each layer; at high magnification, astrocyte somata and processes can be observed (Fig. 1C1 and 1D1).

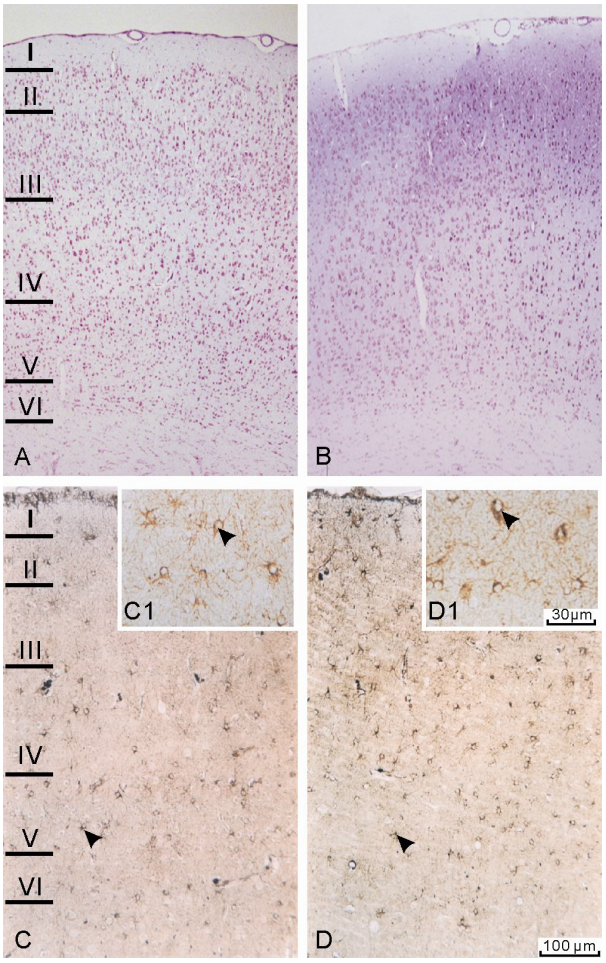

Fig. 1. Neuron and astrocyte density in the cerebral cortex of control (A, C) and ethanol-treated (B, D) rats. (A, B) Nissl staining shows laminar distribution of cortical neurons. (C, D) Astrocyte distribution in cortical layers was detected by GFAP immunoreactivity. (C1, D1) Magnified view of GFAP-positive astrocytes (arrow) revealing characteristic morphology. I, molecular layer; II, outer granular layer; III, outer pyramidal layer; IV, inner granular layer; V, inner pyramidal layer; VI, multiform layer.

A one-way ANOVA revealed that neuronal density differed according to cortical layer in control [$F(5,318) = 227.768$, $P < 0.01$] and ethanol-treated [$F(5,318) = 86.634$, $P < 0.01$] rats, with the lowest density observed in layer I (Fig. 2A). The neuronal density was reduced by 7.83%, 13.85%, 27.77%, 12.06%, 24.71%, and 15.89%, for layers I–VI, respectively, in ethanol-treated rats as compared to controls, with the greatest reductions observed in layers III and V (Fig. 2A).

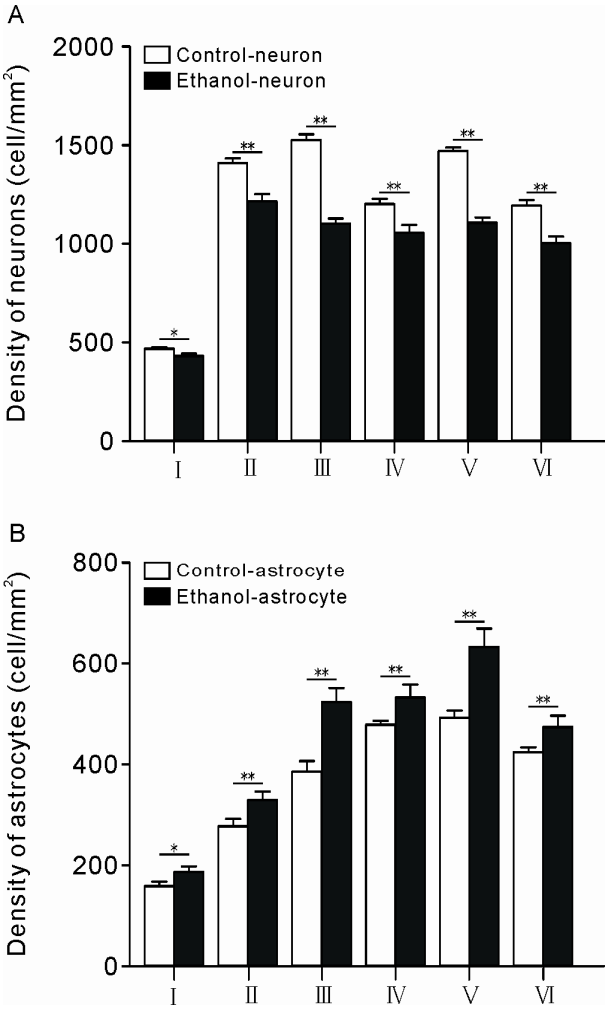

Fig. 2. Quantitative analysis of neuron and astrocyte density in each layer of the cerebral cortex in control and ethanol-treated rats. (A) The mean density of neurons decreased, while (B) the mean density of astrocytes increased in each cortical layer in ethanol-treated rats with respect to controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

Ethanol-induced changes in astrocyte density in the cerebral cortex

As observed for neurons, astrocyte density varied by cortical layer in control [$F(5,318) = 93.125$, $P < 0.01$] and ethanol-treated [$F(5,318) = 42.207$, $P < 0.01$] rats, with the lowest density observed in layer I (Fig. 2B). Astrocyte density was increased by 17.91%, 18.80%, 35.58%, 11.39%, 28.37%, and 11.73% in layers I–VI, respectively, in ethanol-treated rats as compared to controls, with the highest numbers detected in layers III and V (Fig. 2B).

DISCUSSION

Chronic alcohol abuse has adverse effects on brain functioning. In the present study, a significant neuronal loss was observed in the cortex of ethanol-treated rats, consistent with observations in other brain regions (Ikonomidou *et al*., 2000). Notably, the decrease in the number of neurons was dependent on cortical layer, and was highest in layers III and V (Fig. 2), suggesting that neurons in the major output layers are especially sensitive to ethanol-induced toxicity. It has been reported that ethanol-induced hypoxia, accumulation of oxygen free radicals, lipid peroxidation, oxidative stress, and cytotoxin accumulation in tissue may lead to neuronal apoptosis (Ikonomidou *et al*., 2000; West *et al*., 2001; Chauhan *et al*., 2013; Teixeira *et al*., 2014). Moreover, ethanol metabolites such as acetaldehyde can perturb neuronal tubulin polymerization (Jaatinen and Rintala, 2008) and decrease the level of endogenous antioxidants (Scolaro *et al*., 2012), thereby increasing the risk of neuronal apoptosis. Ethanol has also been shown to disrupt neurotransmitter receptor function by blocking glutamate receptors while overactivating GABA receptors and downregulating dopamine receptors (Ramachandran *et al*., 2003; Naseer *et al*., 2014).

The nervous system has various mechanisms for mitigating the effects of toxic substances; one of these is mediated by glia. The parenchyma of the cortex contains many types of glia, with astrocytes being the most abundant. Previous studies have shown that chronic ethanol intoxication stimulates GFAP synthesis that may result in astrocytic hyperplasia (Dalcik *et al*., 2009; Udomuksorn *et al*., 2011). Here it was shown that the number of GFAP-immunoreactive astrocytes was increased in all cortical layers upon ethanol exposure, which was especially obvious in layers III and V, corresponding to the loss of neurons in these layers. We speculate that astrocyte proliferation maybe triggered by and compensate for neuronal death from ethanol-induced toxicity. This may enhance the various functions of astrocytes in the nervous system—such as providing trophic support to neurons, maintaining homeostasis, transferring nutrients, repairing traumatized tissue, modulating synaptic function, and recycling neurotransmitters (Fiacco *et al*., 2009; Allen, 2014)—and thereby mitigate neuronal degeneration so that normal brain functioning can be restored.

In conclusion, the findings of this study demonstrate that a significant number of neurons are lost whereas astrocyte number increases in the cerebral cortex upon chronic exposure to ethanol, with the most obvious differences observed in the major output layers. The loss of neurons may disrupt neural circuitry while increases in astrocyte number may have a compensatory role in maintaining brain function under conditions of ethanol toxicity.

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Conflict of interest statement

The authors have no conflicts of interests.

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