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Chronic Ethanol Administration Causes Expansion of $S100_B$-Immunoreactive Cells in Rat Brain

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1 SUMMMARY

There is increasing evidence that chronic ethanol consumption affects, either directly or indirectly, the survival and viability of cells in the nervous system. This study investigated the effect of long-term ethanol administration on the number of $S100_B$-immunoreactive ($S100_B$-IR) cells in rat cerebral cortex and cerebellum. Anti-$S100_B$ immunohistochemical staining was done at regular timepoints in rats that were administered daily intraperitoneal injections of ethanol (2 g/kg) for 45 days. The analysis showed the presence of $S100_B$-IR cells in all cortical layers and the white matter in both control and ethanol-treated groups. The percentage of $S100_B$-IR cells, however, was found to be highly dependent on the histological position within the cortex and cerebellum. It was observed a significant increase in the number of $S100_B$-IR cells in ethanol-treated rats compared to the control, especially in the output layers of the cortex. Specifically, the following increments were detected-25.03%, 23.81% and 23.23% in cerebral cortical layers III, V and VI, respectively; 30.39% in the Purkinje cell (PC) layer of the cerebellar cortex; 27.68% and 25.00% in the white matter of the cerebrum and the cerebellum, respectively. This study provides the first evidence that chronic ethanol administration stimulates $S100_B$-IR cell hyperplasia in brain, especially in cortical output layers and the white matter. The expansion of $S100_B$-IR cells in response to ethanol insult in the brain is likely to protect injured tissues and partly contribute to maintaining neuronal function.

2 INTRODUCTION

$S100_B$ is expressed in a wide range of cells in the central nervous system, especially glial cells (Heizmann *et al.,* 2002; Donato *et al.,* 2013). It has been implicated in numerous intracellular and extracellular functions, such as suppression of protein kinase C (PKC)-mediated phosphorylation, regulation of Ca^{2+} homeostasis, inhibition of microtubule assembly, and, regulation of enzymatic activity, cell cycle progression and inflammatory response (Azmitia

et al., 1990; Heizmann *et al.,* 2002; Donato *et al.,* 2013). Additionally, in the developing brain, $S100_B$ also acts as a neurotrophic factor and a neuronal survival protein crucial for neuronal maturation (Azmitia *et al.,* 1990; Van Eldik and Wainwright, 2003; Donato *et al.,* 2013). Its expression is markedly elevated under a variety of conditions, such as senescence (Baydas and Tuzcu, 2005), brain trauma (Yardan *et al.,* 2011; Zongo *et al.,* 2012), neurotoxicity (Baydas and Tuzcu, 2005), neuroinflammation (Beer *et al.,* 2010; Guerra *et al.,* 2011; Tang *et al.,* 2011) and neurological diseases like Alzheimer's disease (Tang *et al.,* 2011; Yardan *et al.,* 2011), Parkinson's disease (Yardan *et al.,* 2011; Sathe *et al.,* 2012; Guo *et al.,* 2013), epilepsy (Calik *et al.,* 2013) and melanoma (Nonaka *et al.,* 2008). Hence, the level of expression of $S100_B$ could be indicative of the presence of injuries in the nervous system. It is known that chronic ethanol consumption causes neurotoxic effects, evidence of which has been obtained from molecular, cellular, histological and behavioural studies (Servais *et al.,* 2005; Dlugos, 2006a; Garcia-Valdecasas-Campelo *et al.,* 2007; Jiang *et al.,* 2008; Stephens and Duka, 2008; Mah *et al.,* 2011). However, there is some evidence suggesting that in response to ethanol insults, the nervous system might activate a compensatory mechanism against neurotoxicity. For example, chronic ethanol exposure significantly enhances the activity of glial cells that play an important role in maintaining neural homeostasis (Baydas and Tuzcu, 2005; Donato *et al.,* 2013). $S100_B$ is synthesized and secreted mainly by glial cells. However, whether and how $S100_B$ expression is modified in response to ethanol toxicity remains unclear. The current study is aimed at investigating a possible correlation between ethanol-induced neurotoxicity and $S100_B$ expression in rat brain. Our experiments demonstrate an increase in $S100_B$ expression in response to ethanol insult in the output layers and white matter of the cortex and cerebellum. Our data will facilitate further research into the mechanism underlying ethanol-related brain injuries.

3 MATERIALS AND METHODS

3.1 Animals: Ten 2-month old male Sprague–Dawley (SD) rats, weighing 220–250 g, were randomly assigned into two groups. The experimental group (n = 5) received intraperitoneal injections of ethanol (2 g/kg body weight dissolved in 1 ml saline; the injection period lasted 30 s) once each day for 45 consecutive days; the control group (n = 5) received only saline treatment (1 ml 0.9% NaCl). The same injection protocol was used in both groups. The animals were monitored daily and weighed weekly in order to evaluate their health status, and possible toxic side-effects from the procedure. All animals were maintained individually in a temperature-controlled (22±2 ℃) house with a defined light/dark-cycle (12 h/12 h), and food and water ad libitum. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2 Tissue preparation: After treatment, rats were anesthetized using sodium pentobarbital (40 mg/kg, intraperitoneal injection). Once the reflexes were abolished, 100 ml of 0.9% NaCl containing 50 mg heparin was perfused through the left ventricle, followed immediately by 100 ml of fixative solution containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). After perfusion, the skull was opened, and the primary motor area (M1) of the cerebral

cortex and the cerebellar vermis were dissected out according to the coordinates obtained from a rat brain atlas (Swanson 1998). After further fixation overnight in the same fixative, blocks were trimmed and washed in PBS, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Consecutive 6 µm thick coronal sections were cut and mounted on 3-Aminopropyl-Triethoxysilane (APES; Sigma, USA; 1:50 in acetone)-coated microscopic slides for immunostaining.

3.3 . Immunostaining: On each slide, a series of sections approximately 120 µm apart were mounted and assayed for expression of $S100_B$ by immunostaining, according to a well-established protocol. Briefly, sections were deparaffinized in xylene, hydrated through an ethanol gradient series ending with distilled water. The sections were then incubated in 3% H_2O_2 for 5 min at RT to quench endogenous peroxidase activity, followed by 5% goat serum for 10 min at RT to suppress non-specific antibody binding. Next, sections were incubated in anti-$S100_B$ antibody (rabbit polyclonal; 1:500; Sigma, USA) for 1 h at 37℃, followed by biotinylated anti-rabbit IgG (1:100; Sigma, USA) for 10 min at 37℃. They were then treated with preformed avidin-biotin-peroxidase complexes (Sigma, USA) for 10 min at 37 ℃. Finally, antibody binding was visualized by incubating sections in a substrate for peroxidase enzyme, 0.05% 3,3'-diaminobenzidine (DAB)/0.01% hydrogen peroxidase (Sigma, USA) for 10 min at RT. After dehydrating through an ethanol gradient and clearing the sections in xylene, the slides were mounted in permount. Negative control immunostaining was performed on adjacent sections by substituting the primary antibody for PBS.

3.4 . Quantification of $S100_B$-immunoreactive ($S100_B$-IR) cell measurements: Semi-quantitative measurements of cell number were made under the microscope, blinded to the sample identities. At a magnification of 400X, the number and density (cells/mm^2) of $S100_B$-IRcells in each cortical layer and in the subcortical white matter of cerebral cortex (Fig. 1) and cerebellum (Fig. 3) was calculated in each slide in 3 randomly chosen visual fields using a calibrator (125 µm × 125 µm). In the Purkinje cell (PC) layer of the cerebellum, the number of $S100_B$-IR cells and their linear density (cells/mm) was calculated using an eyepiece micrometer (125 µm). Only those cells where a yellow-stained soma could be clearly seen above the background were considered as $S100_B$-IR cells.

3.5 . Quantitative Analysis: All data are represented as Mean ± S.E.M. Statistical significance was evaluated by Student's *t*-test or a two-way analysis of variance (ANOVA), and a *P*-value < 0.05 was considered significant.

4 RESULTS

4.1 . Physiological changes following ethanol administration: Soon after ethanol treatment, rats showed multiple symptoms of alcoholism, including narcoma, tachycardia, twitch and kinetic ataxia, all of which disappeared after a few hours. These symptomatic changes were inconsistent with a previous report where the peak ethanol concentration was achieved about 40 min after injection, and which returned to baseline approximately 4–5 h post-injection (Yoshimoto and Komura, 1993). Over 45 days of ethanol administration, the body weight showed a slight tendency to decrease when compared to the control group, but this difference was not statistically significant (data not shown).

4.2 . Ethanol-induced changes in $S100_B$-IR cell density in the cerebral cortex: Between the pial surface and the white matter, the cerebral cortex can be histologically divided into six layers: molecular

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layer (I), outer granular layer (II), outer pyramidal layer (III), inner granular layer (IV), inner pyramidal layer (V), multiform layer (VI) and the subcortical white matter (WM) (Swanson 1998). Sections from both experimental and control groups that were assayed for $S100_B$ expression by immunostaining, revealed brown-yellow staining of the soma, of different sizes and shapes in all cortical layers (Fig. 1 a and b). A cursory comparison of the immunostaining results in the two groups indicated a notable enlargement in soma size and an obvious increase in staining intensity in the $S100_B$-IR cells of ethanol-treated rats in comparison to the controls (Fig. 1 a1, a2 and a3 *vs* b1, b2 and b3).

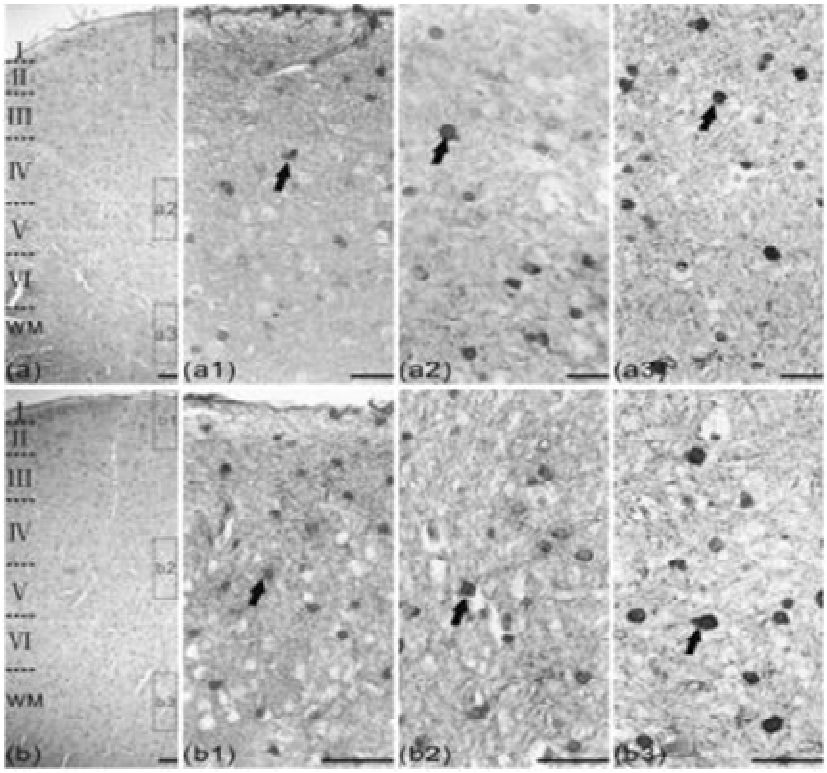

Figure 1: $S100_B$-immunostaining of the cerebral cortex. Both control (a, a1, a2 and a3) and ethanol-treated cortical sections (b, b1, b2 and b3) show distinct $S100_B$-IR cells (arrows) in each cortical layer (I-VI) and the subcortical white matter. The layer-specific distribution of $S100_B$-IR cells in the cortex is depicted as follows: overall distribution (a and b), superficial cortical layers (a1 and b1), deep cortical layers (a2 and b2), and subcortical white matter (c1 and c2). Abbreviations: I- molecular layer; II- outer granular layer; III- outer pyramidal layer; IV- inner pyramidal layer; V- inner granular layer; VI- multiform layer, WM- white matter. Scale bar = 20 μm.

Statistical analysis showed that in both control and experimental groups the density of immunostaining was highly dependent on the precise histological location (cortical layer), with the least density in Layer I and the maximum density in the white matter [main effect of histological location: $F(6,133) = 16.674$, $P< 0.05$ in ethanol-treated rats; $F(6,133) = 9.540$, $P< 0.05$ in the controls] (Fig. 2). A significant increase in the density of $S100_B$-IR cells, in all cortical layers and the white matter was found in experimental rats

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compared to the controls [main effect of ethanol: $F(1,38) = 85.900$, $P< 0.05$]. Between Layer I and the white matter, the mean density of $S100_B$-IR cells in ethanol-treated rats compared to control rats, was higher by 16.39%, 17.72%, 25.03%, 16.22%, 23.81%, 23.23% and 27.68%, respectively. This increase appeared to be more severe in Layers III, V, VI and the white matter (Fig. 2).

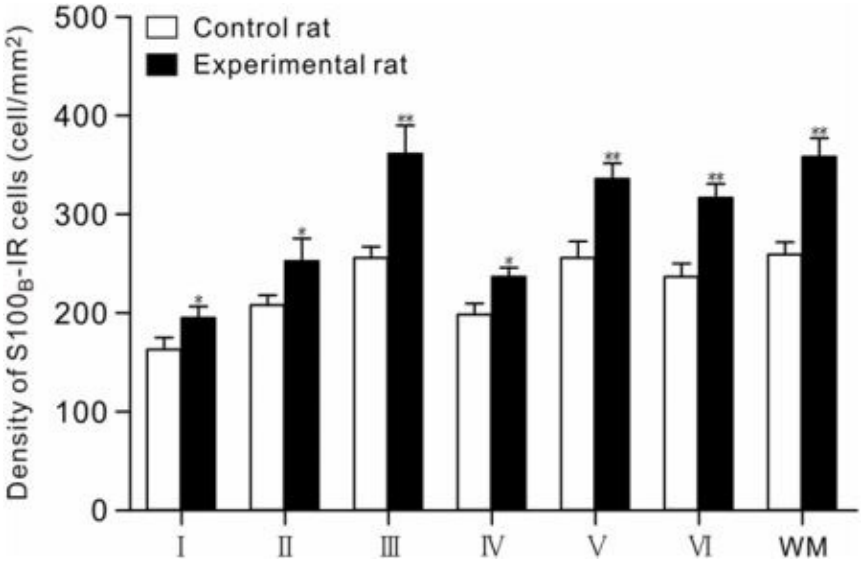

Figure 2: The mean density of $S100_B$-IR cells in the cerebral cortex. Shown here is a comparison of the mean density of $S100_B$-IR cells in ethanol-treated versus control rats within each cortical layer.$^{*}P < 0.05$; $^{**}P < 0.01$.

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4.3 . Ethanol-induced changes in S100B-IR cell density in the cerebellum: The cerebellum is divided into the following histological layers: the molecular layer, PC layer, granular layer and the subcerebellar white matter (Swanson 1998). S100B-IR cells were abundantly located in all layers except for the molecular layer (Figures 3 a and b). A cursory comparison of the immunohistochemical staining in the control and experimental groups indicated a notable increase in soma size and staining intensity in the S100B-IR cells of ethanol-treated rats versus the control group (Fig. 3 a1 and a2 *vs* b1 and b2).

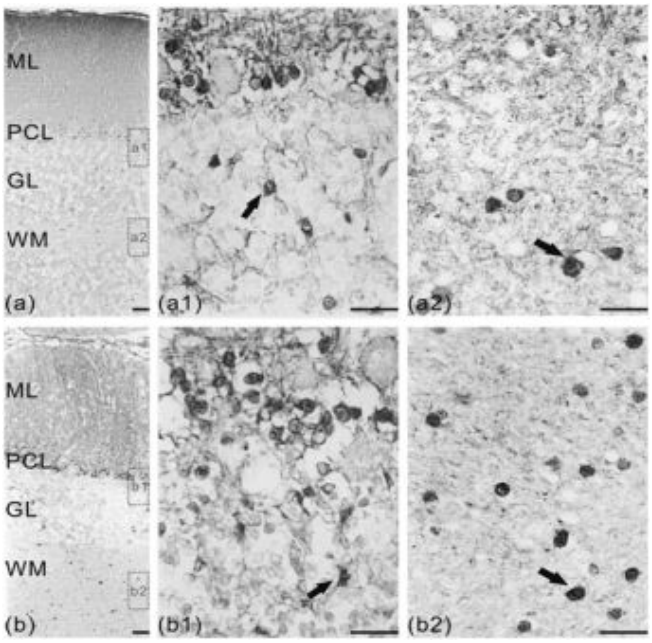

Figure 3: S100B-immunostaining in the cerebellum. S100B-IR cells (arrows) can be seen in all layers in both control (a, a1 and a2) and ethanol-treated rats (b, b1 and b2). The layer-specific distribution of S100B-IR cells is depicted as follows- overall distribution (a and b), PC and granular layer (a1 and b1), subcerebellar white matter (a2 and b2). Note the absence of S100B-IR cells in the molecular layer. Abbreviations: ML- molecular layer, PCL- Purkinje cell layer; GL- granular layer; WM- white matter. Scale bar = 20 μm.

Further analysis revealed that in both groups, the density of S100B-IR cells was highly dependent on their precise location within the cerebellum, with least density in the granular layer and maximum density in the PC layer and the white matter [main effect of histological location: $F(2,57) = 45.123$, $P < 0.05$, in ethanol-treated group; $F(2,57) = 48.324$, $P < 0.05$, in the control] (Fig. 4). We found a significant increase in the density of S100B-IR cells, in all layers in ethanol-treated rats when compared to the controls [main effect of ethanol: $F(1,38) = 82.437$, $P < 0.05$]. The mean density of S100B-IR cells in ethanol-treated rats was higher than controls by 30.39%, 17.59% and 25.00% in the PC layer, granular layer and the white matter, respectively. The S100B-IR cells in PC layer and the white matter appeared to be the most proliferative (Fig. 4).

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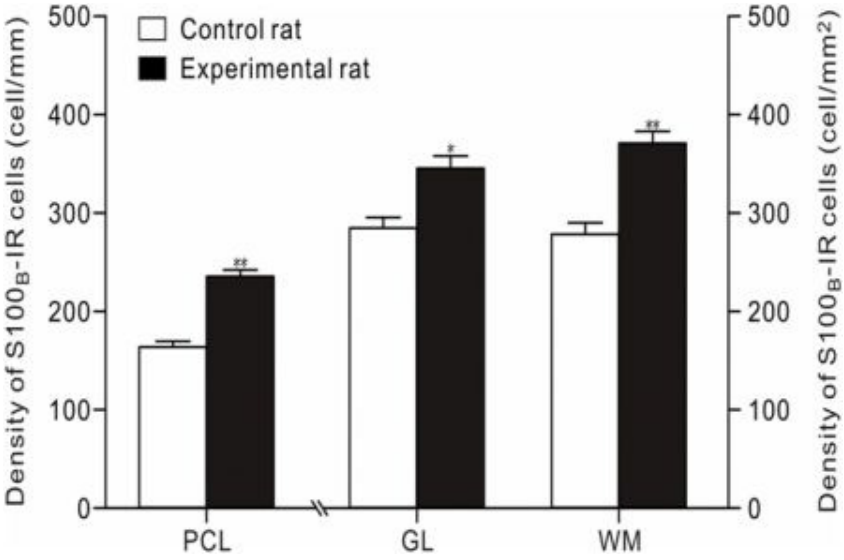

Figure 4: The mean density of $S100_B$-IR cells in the cerebellum. Shown here is a comparison of the mean density of $S100_B$-IR cells in ethanol-treated versus control rats in each layer of the cerebellum. $^{*}P < 0.05$; $^{**}P < 0.01$.

5 DISCUSSION

Chronic consumption of ethanol has been shown to cause adverse effects on brain function, resulting in physiological impairment in both humans and experimental animals, including conditions such as motor disorder, perception disturbance, neuropsychological damage and even dementia (Nordmann, 1994; Servais *et al.,* 2005; Stephens and Duka, 2008; Shirpoor *et al.,* 2009). Although the precise mechanism underlying these neurotoxic effects is unclear, several factors have been proposed to contribute to the neurotoxicity, including ethanol-related brain atrophy (Garcia-Valdecasas-Campelo *et al.,* 2007), retrogression of neuronal configuration (Dlugos, 2006a; Dlugos, 2006b; Dlugos, 2008; Jiang *et al.,* 2008), and alterations in the electrophysiological properties of neurons (Servais *et al.,* 2005; Luo, 2012). More importantly, ethanol has been found to alter the chemical composition of neurons, leading to increased oxidative stress to lipids, DNA, and proteins, and decreased levels of protective endogenous antioxidants (Nordmann, 1994; de la Monte *et al.,* 2000; Pirlich *et al.,* 2002; Baydas and Tuzcu, 2005). Ethanol has also been shown to subvert the ratio of pro-apoptotic and anti-apoptotic proteins in favour of cell death (de la Monte *et al.,* 2000). All these are crucial elements in ethanol-dependent neurotoxicity. Remarkably, the nervous system has inherent compensatory mechanisms to partially combat neurotoxicity. One such important mechanism is mediated by glial cells that appear to be highly sensitive to ethanol insult (Acarin *et al.,* 1999; Baydas and Tuzcu, 2005). Consequently, they play an active role in maintaining function during ethanol toxicity (Acarin *et al.,* 1999; Baydas and Tuzcu, 2005). $S100_B$ is a glial protein. The current study provides the first direct evidence that chronic ethanol administration can be correlated to an increase in $S100_B$ expression in the brain. In this study, we have demonstrated an increase in the density of $S100_B$ cells in both the cerebral cortex and the cerebellum in

ethanol-treated rats. These results, however, are inconsistent with a previous report where chronic ethanol consumption in rats was shown, through western blotting, to significantly increase $S100_B$ expression in the hippocampus (Baydas and Tuzcu, 2005). A recently proposed hypothesis that is widely prevalent is that the effects of ethanol on brain function are partly due to the altered activity of ion channels, especially the calcium-permeable ion channels, which in turn affect synaptic transmission (Wynne *et al.,* 2009; Mah *et al.,* 2011). Since $S100_B$ is a Ca^{2+}-binding protein that can regulate both intracellular and extracellular Ca^{2+} ion equilibrium (Kligman and Marshak, 1985; Fano *et al.,* 1995; Lin *et al.,* 2004), we speculate that the increased $S100_B$ expression in ethanol-exposed brain functions to revert the aberrant Ca^{2+}ion concentration towards the equilibrium and hence partly restore neuronal function. Our observations also revealed an increased proliferative tendency of $S100_B$ cells in cortical output layers and the white matter in response to ethanol toxicity. This suggests that these specific areas might require increased activation of compensatory mechanisms in order to maintain function.

6 CONCLUSIONS

Our present study reveals that chronic administration of ethanol markedly increases the number of $S100_B$-IR cells in the rat brain and that their concomitant proliferation seems to be higher in the output layers and the white matter. The enhanced $S100_B$ expression in brains affected by ethanol toxicity may play a compensatory role in maintaining neuronal function in the injured nervous tissues.

7 ACKNOWLEDGEMENTS

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