Ethanol down regulates the expression of myelin proteolipid protein in the rat hippocampus

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Abstract: It is well known that chronic ethanol treatment affects the synthesis of RNA and protein in the brain and the maintenance and function of nervous system. The changes in myelination-related genes are most prominent in human alcoholics. Previously, our cDNA microarray study showed altered Proteolipid protein (PLP), a major protein of central myelin. The present study aimed to gain more understanding of the expression of PLP after chronic ethanol treatment. Male Sprague-Dawley rats were daily treated with ethanol (15% in saline, 3 g/kg, i.p.) or saline for 14 days. Messenger RNAs from hippocampus of each group were subjected to cDNA expression array hybridization to determine the differential gene expressions. Among many ethanol responsive genes, PLP was negatively regulated by ethanol treatment, which is one of the most abundant proteins in the CNS and has an important role in the stabilization of myelin sheath. Using northern blot and immunohistochemical analysis, we showed the change in expression level of PLP mRNA and protein after ethanol treatment. PLP mRNA and protein were decreased in hippocampus of rat with chronic ethanol exposure, suggesting that ethanol may affect the stabilization of myelin sheath through the modulation of PLP expression and induce the pathophysiology of alcoholic brain.

Key words: rat, brain, hippocampus, alcohol, proteolipid protein

Introduction

Chronic ethanol consumption has adverse effects on the entire central nervous system (Ollat & Sebban, 1983; Deitrich et al., 1989; Samson & Harris, 1992). Previous studies have demonstrated that chronic ethanol intake induces alteration of brain functions, which may be caused by effects of ethanol on neuronal functions (Walker et al., 1980; Deitrich et al., 1989; Ryabinin 1998). Changes in neuronal functions caused by chronic exposure to ethanol are believed to underlie manifestations of a variety of psychiatric disorders and behavioral dysfunctions (Deitrich et al., 1989; Samson & Harris, 1992).

Recent study of gene expression monitoring with cDNA expression arrays provides a simple way to explore the biochemical effects of ethanol on the brain (Duggan et al., 1999; Bowers et al., 2006). The results of cDNA microarray studies showed that various genes related to oxidative stress and membrane trafficking were changed by chronic ethanol treatment. Also, these studies showed that ethanol interacts with various proteins and modifies protein function in the central nervous system (CNS) (Saito et al., 2002). Specially,

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the changes in myelination-related genes were most prominent in human alcoholics (Liu *et al.*, 2004). One of the most abundant protein in mammalian CNS is Proteolipid protein (PLP), and PLP contributes to myelin's unique biological properties owing its high affinity to lipid (Boison *et al.*, 1995; Möbius *et al.*, 2008). Consistently, we detected PLP changes in our cDNA microarray study.

The aim of the present study is to investigate the changes in PLP mRNA and protein expression in the hippocampus of rat with prolonged ethanol treatment, which may have an important role in learning and memory.

Materials and Methods

Experimental animals

Male Sprague-Dawley rats were purchased from Samtaco Co. (Osan, South Korea) and were maintained in the animal facility at the Gyeongsang National University School of Medicine. Rats were treated in accordance with standard guidelines for laboratory animal care. All rats were provided standard chow and water *ad libitum*. Ethanol was diluted to 15% and given intraperitoneally at a dose of 3 g/kg b.w. in solution of 0.9% saline for 14 days. We used total 10 animals per group, 6 animals for extraction of RNA and 4 animals for tissue preparation. Control animals were injected with an equivalent volume of 0.9% saline. This dose resulted in blood ethanol levels that peak within 15 min of administration via either route (intraperitoneal, intragastric) and remain elevated for at least 3 hr.

cDNA expression array

Gene expression was analyzed using a cDNA expression array (Clontech laboratory, Palo, CA). Twenty μg of total RNA from hippocampus of saline- and ethanol-treated animals were reverse transcribed respectively. Solution containing hippocampal RNA, oligo (dT) primer, 0.5 μM dNTP (without dCTP), and reverse transcription buffer were heat-denatured at 65°C for 10 min. 50 μCi of 32P-d CTP (3,000 Ci/mmol, NEN), 200 units of MMLV reverse transcriptase (Promega, Fitchburg, Wisconsin) and 20 units of RNasin were added and incubated at 37°C for 1 hr. Free isotope was removed by Sephadex G-50 column chromatography (Pharmacia, Uppsala, Sweden). Radioactivity was checked by β-counter. Labelled probes were denatured by boiling for 10 min and added to hybridization solution containing 50% deionized formamide, 5X SSPE, 1X Denhardts solution, 0.1% SDS, and

2 mg of heat-denatured salmon sperm DNA, to reach a final probe concentration of 1×10^6 cpm/ml and freshly applied to the atlas cDNA expression array membrane, which was prehybridized in 20 ml of hybridization solution at 42°C on a compact locker for 2 hr. Hybridization proceeded overnight at 42°C on a compact locker. Following hybridization, membranes were stringently washed with agitation for 10 min in 200ml of prewarmed (42°C) solution 1 (2X SSPE, 0.1% SDS) twice and solution 2 (0.1X SSPE, 0.1% SDS) twice at 42°C for 5 min. Afterwards, membranes were exposed to X-ray film (Pharmacia, Uppsala, Sweden) at −70°C for 3 days.

Tissue preparation

For *in situ* hybridization & immunohistochemistry, rats were anesthetized with pentobarbital sodium and perfused via the left cardiac ventricle with approximately 250~300 ml of 4% paraformaldehyde in 0.1 M Phosphate buffered saline after perfusion with 100~150 ml of 0.9% saline. Brains were quickly removed after perfusion and further fixed with the same fixative for 12 hr at 4°C, fixed brains were rinsed for 24 hr in 20% sucrose and sectioned serially at 12 μm thickness in a cryostat (Leica, Wetzlar, Germany) at −20 μm. Each section was then mounted on gelatin coated slides and stored at −70°C until use. Since prolonged storage of tissue section seemed to decrease the intact mRNA, tissue section was used within 1 month. Solutions were prepared with DEPC-treated distilled water to further eliminate RNase contamination.

Northern blot analysis

Total RNAs were extracted by the acid guanidinium thiocyanate-phenol-chloroform method. The total RNAs (20 μg) separated by formaldehyde/1.2% agarose gel electrophoresis, transferred to a 0.45 μm Nytran membrane for 18~24 hr by diffusion blotting, then the RNA blotted membrane was UV-crosslinked. Prehybridization was carried out at 42°C for 2 hr in a heat-sealable plastic bag (Invitrogen, Carlsbad, CA) with hybridization buffer consisting of 50% deionized formamide, 5X SSPE, 1X Denhardts solution, 0.1% SDS, and 2 mg of heat-denatured salmon sperm DNA. After addition of each ^{32}P-labelled PLP cDNA probe as made by random primer labeling method. Hybridization was preformed at 42°C for 24 hr. Following hybridization, the membrane was washed twice with 2x SSPE and 0.1% SDS at room temperature for 10 min, followed by the second washing with 0.07x SSPE, 0.5% SDS, and 5 mM EDTA (pH
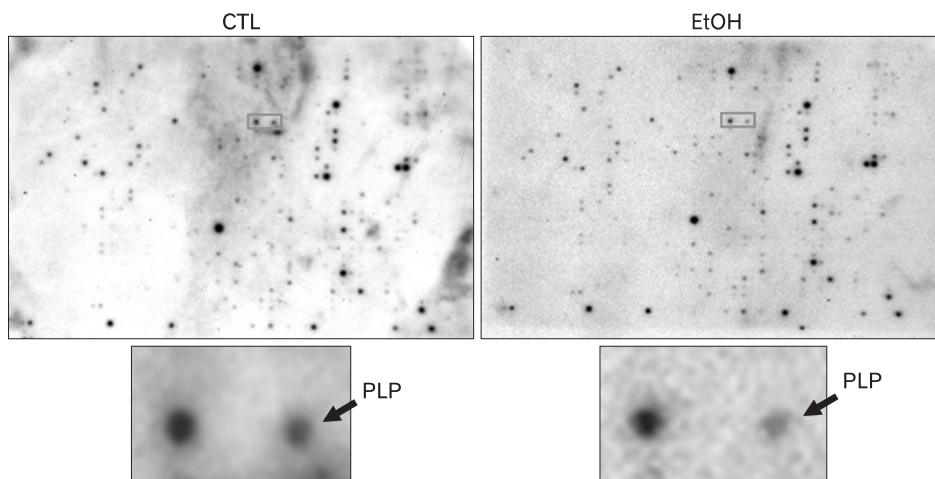
CTL

EtOH

Fig. 1. Expression array hybridized with ^{32}P-labeled cDNA from hippocampal pools of control saline- and ethanol-treated animals as revealed with X-ray film (upper panel) exposed for 3 days. The cDNAs on the bottom line of upper panel are commonly considered as housekeeping genes. Arrows in the magnified images indicate the cDNA of PLP which was obviously reduced in ethanol-treated group compare to control group.

8.0) at 42°C for 5 min. The membrane was exposed to X-ray film (Fuji, Tokyo, Japan) at −70°C for 3 days.

In situ hybridization

All solutions were made with sterile water, and glasswares were autoclaved to prevent contamination by RNase. The antisense PLP cRNA was transcribed *in vitro* from a vector containing a 238 bp fragment of the cloned rat cDNA using sp6 polymerase in the presence of ^{35}S-UTP (1,250 ci/mmol, NEN). ^{35}S-UTP labeled probes were prepared using *in vitro* transcription kit (Promega, Fitchburg, Wisconsin). Sections were dried, washed with 0.1 M Phosphate buffered saline, permeabilized by proteinase K, acetylated, and hybridized at 55°C for 20 hr. The sections were then incubated with 50 μg/ml RNase A for 30 min at 37°C and then washed with a series of SSC solutions. The highest stringency wash was 0.1 X SSC at 60°C for 30 min, and dehydrated in alcohol solution with ascending concentration. Tissue Sections were coated with NTB2 emulsion (Invitrogen, Carlsbad, CA), kept at 4°C for 14 days, and developed. Tissue sections were counterstained with cresyl violet. The slides were observed under a bright field microscopy, and then photographed.

Immunohistochemistry

For the localization of immunoreactive PLP, avidin-biotin complex (ABC) method was used. Tissue sections on slides were air-dried, dipped in 0.1 M PBS (pH 7.0) twice for 10 min, and rinsed with Triton X-100 in 0.1 M PBS for 10 min. Tissue sections were incubated with 50 μl of normal horse serum, diluted 1 : 100 for 30 min to exclude the nonspecific binding before the primary antibody application. Then slides were applied with 50 μl of the primary antibody, mouse-
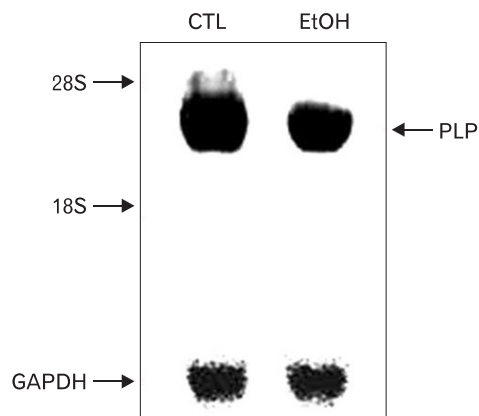

Fig. 2. Northern blot analysis of PLP mRNA. Twenty μg of total RNA from hippocampus of saline- or ethanol-treated rats were electrophoresed formaldehyde contained 1.2% agarose gel and transferred to nytran membrane, then hybridized with ^{32}P-labeled gene specific cDNA probe. Membrane was exposed to X-ray film for 24 hr at −70°C. CTL, saline-treated control group; EtOH, ethanol-treated group; 28S, 28s ribosomal RNA; 18S, 18S ribosomal RNA; PLP, proteolipid protein. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

derived anti-PLP (Santa Cruz Biotechnology, CA) with a final dilution of 1 : 500 for overnight at 4°C. Tissue sections were washed with 0.1 M PBS for 10 min twice, incubated with biotinylated secondary antibody (Santa Cruz Biotechnology, CA) for 2 hr at room temperature. After washing an excess secondary antiserum, ABC diluted 1 : 250 was treated for 2 hr at room temperature. Slides were washed again in PBS and Triton X-100, and then were rinsed with PBS for 10 min at room temperature. Slides were incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in PBS for 10 min. Hydrogen peroxide was added to the same DAB

solution to make a final concentration of 0.01% H_2O_2, and the container was shaken gently as the reaction proceeded. After 1~2 min, as determined by the degree of staining with the light microscope, the reaction was terminated with several washes of PBS. Tissue sections were observed under a light microscopy and photographed.

Results

To examine the effects of ethanol on PLP expression in the hippocampus, we performed cDNA microarray in SD rats, and found that PLP was reduced approximately ~36% (Fig. 1). In order to confirm the reduced expression of PLP by ethanol using array hybridization, total RNAs were extracted from hippocampus and subjected to northern blot analysis with GAPDH as internal standard (Fig. 2). Quantification of PLP mRNA northern blot analysis showed that PLP mRNA was decreased approximately ~30% in the hippocampus of ethanol-treated rats compared with control and this result was consistent with cDNA array.

To evaluate the localization and expressional changes of PLP mRNA in the hippocampal region, in situ hybridization was applied. PLP mRNA was observed in the hippocampus proper (stratum radiatum layer) where the axons, from pyramidal cell and inter neuronal dendrite, form network (Fig. 3). Higher magnification view of PLP in saline treated group
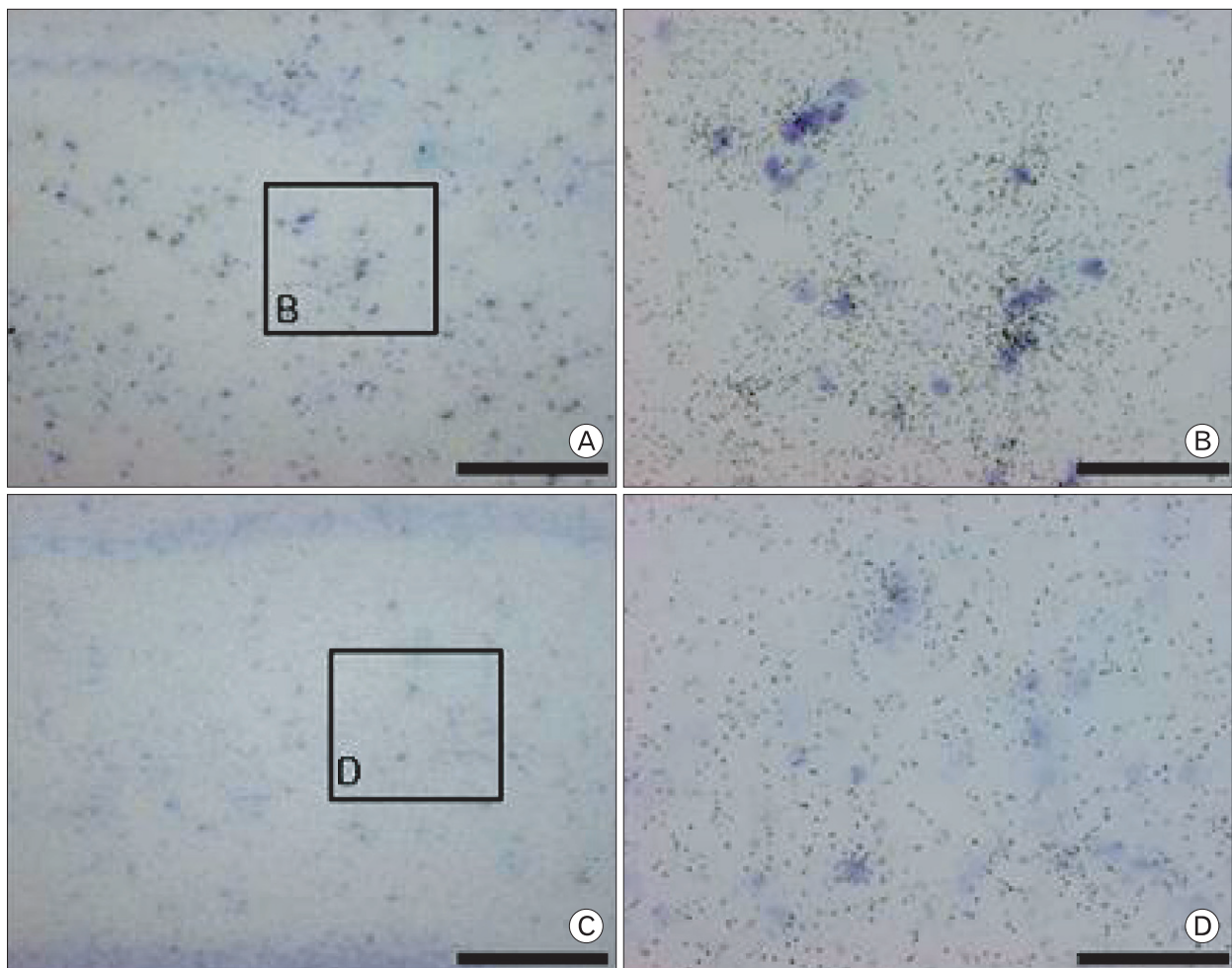

Fig. 3. Bright field photomicrography of PLP mRNAs in the hippocampus of control saline- (A, B) and ethanol-treated animals (C, D). PLP mRNAs were localized in the brain slice (12 μm in thickness) at the level of 3.0 mm post bregma using *in situ* hybridization with ^{35}S-UTP labeled anti-sense PLP cRNA probe. Slides were dipped into NTB2 emulsion (Kodak Co., New York, USA), exposed at 4°C for 2 weeks, developed in Kodak D19 developer (Kodak Co., New York, USA) at 15°C and counterstained with crezyl violet. Cells had positive signals against PLP mRNA were observed in control saline-treated group (A, B) but those were reduced in hippocampal area of ethanol-treated group (C, D). Scale bars, A and C, 200 μm; B and D, 50 μm.
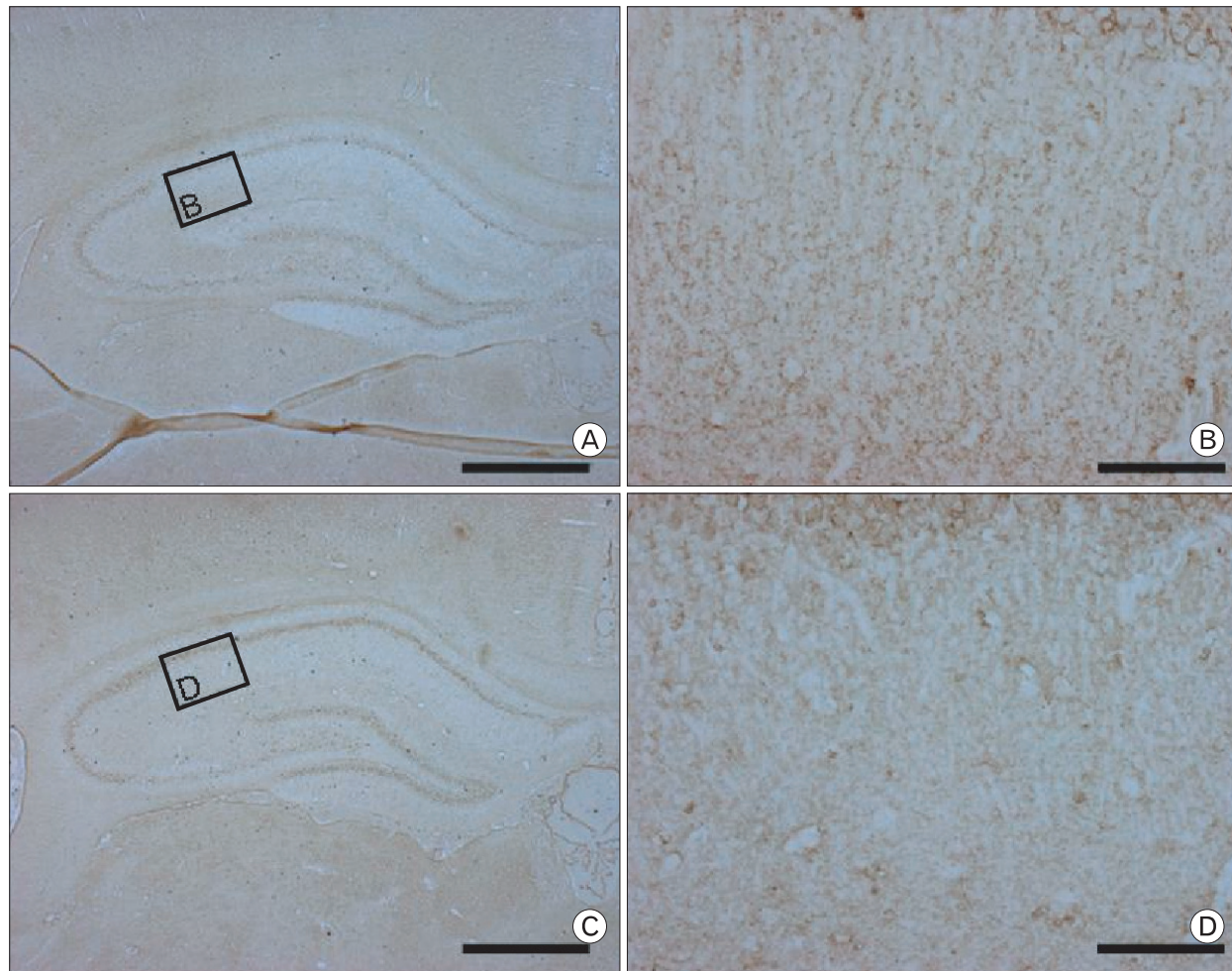
Fig. 4. Immunohistochemical detection of myelin PLP in the rat hippocampus from saline- (A, B) and ethanol-treated rats (C, D). Clear-cut decreases in PLP immunoreactivities were observed in the ethanol-treated hippocampal regions. Scale bars=A and C, 1,000 μm; B and D, 100 μm.

(Fig. 3B) showed some cells have dense signals but not in the same area of ethanol-treated group (Fig. 3D). Since PLP is the main protein of CNS myelin and myelin sheaths are formed by oligodendrocyte (Boison *et al.*, 1995; Sarret *et al.*, 2010), those cells may be oligodendrocytes. As well, PLP is known to colocalize with myelin basic protein , supporting our results (Boison *et al.*, 1995).

It was clearly confirmed that the levels of PLP mRNA expression decreased in the hippocampus of ethanol-treated rats using northern blot analysis, and *in situ* hybridization.

Then, we examined whether ethanol affects PLP using immunohistochemical analysis. We found that PLP in the hippocampus from ethanol-treated rats was reduced in the hippocampus stratum radiatum layer compared with control (Fig. 4).

Discussion

In the present study, we found that PLP mRNA and its protein expression was reduced in the hippocampus of rats with prolonged ethanol administration. This result augmented that hippocampus is one of the brain regions most sensitive to prolonged ethanol administration and suggested that chronic ethanol administration may interfere normal function of hippocampus primarily via down-regulating the PLP mRNA and its protein expression.

In CNS, myelin is a modified form of the oligodendroglial plasma membrane, and spiraled around the axon extending to form a multilamellar structure. The compaction of myelin is essential for neuronal function, and it involves the close apposition of the cytoplasmic and extracellular surfaces of

plasma membrane from specialized glial cells to form the major dense line (MDL) and the intraperiod line (IPL), respectively (Inoue *et al.*, 1973; Peters *et al.*, 1991). The myelin PLP is a main component of intraperiod lines and its function is to assemble myelin lamellae (Boison & Stoffel, 1994; Boison *et al.*, 1995). Although the full array of biological functions of the myelin proteolipid has not yet been clearly defined, PLP appears to be important to stabilize the myelin sheath and maintain the structural integrity of the myelin membrane (Duncan *et al.*, 1987; Boison & Stoffel, 1994).

Myelination of nerve fibers is essential for the function of the vertebrate nervous system. Myelination in the brain has been an area of interest in human alcohol studies due to the observed brain damage in alcoholics and the potential role of demyelination in this process (Kril & Halliday, 1999; Lewohl *et al.*, 2001). It was reported that myelin-related genes, myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin oligodendrocytes glycoprotein (MOG) were down-regulated in the frontal cortex of human alcoholics (Liu *et al.*, 2004). Also, the compaction of myelin enlamellate in the CNS of PLP-null mice was incompleted (Rosenbluth *et al.*, 2006).

A series of mutant animals lacking PLP (Boison *et al.*, 1995; Rosenbluth *et al.*, 1996; Klugmann *et al.*, 1997) as well as several human disorders involving the PLP gene (Griffiths *et al.*, 1998; Daigo *et al.*, 2008; Tanaka *et al.*, 2009) display severe neurological impairment, emphasizing the physiological importance of this protein in nervous system. In the absence of myelin sheath, as seen in demyelization diseases, impulse conduction is impeded resulting in severe sensory and motor deficits (Boison & Stoffel, 1994).

Although PLP expression and its function in the cortex and cerebellum were widely studied in human alcoholics and murine alcohol model, PLP in the hippocampus remains to be explored. In the present study, we showed the expression of PLP mRNA and its protein were reduced in the hippocampus of rats with chronic ethanol treatment. More research is needed to elucidate the exact mechanism of deleterious effects of ethanol on the hippocampus, but one possible scenario is that prolonged ethanol exposure may interfere the normal function of hippocampal neurons by down-regulating the PLP expression.

Acknowledgments

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