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Proteome Analysis of the Cerebellum Tissue in Chronically Alcohol-Fed Rats

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Abstract

Background: Cerebellar degeneration is one of the most common effects of chronic alcohol exposure, and Purkinje cells are the main targets of alcohol-induced cerebellum neuropathology, but the underlying mechanism remains unclear.

Methods: Eight rats were fed for 8 weeks with a nutritionally adequate liquid diet containing either ethanol as 36% of the total caloric content or an isocaloric control diet. Rat cerebellum homogenates were subjected to agarose two-dimensional electrophoresis (2-DE), and the protein expression profiles in chronically alcohol-fed rats and the pair-fed controls were compared. The observed changes in the protein expression levels were confirmed using immunoblotting analysis.

Results: Three protein spots changed significantly in intensity according to 2-DE. Based on immunoblotting analysis, low expression levels of microtubule-associated protein-2 (MAP2) and the overexpression of voltage-dependent anion channel protein 1 (VDAC1) were observed in the cerebellum of alcohol-fed rats. The expression levels of both proteins did not change in other parts of the brain.

Conclusions: Low expression levels of MAP2 and overexpression of VDAC1 were detected using proteome analysis of the cerebellum tissue from chronically alcohol-fed rats. Changes in the expression of these proteins may be related to cerebellar degeneration following chronic alcohol consumption.

Keywords: Cerebellum; Chronically alcohol-fed rats; Proteome analysis

Abbreviation: IEF: Isoelectric Focusing; 2-DE: 2-Dimensional Electrophoresis; SDS-PAGE: Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis; IPG: Immobilized pH Gradient; CBB: COOMASSIE Brilliant Blue; CAN: Acetonitrile; MS: Mass Spectrometry; HPLC: High Performance Liquid Chromatography; TBS-T: Tris Buffere Saline-0.1% Tween 20; MAP2: Microtubule-Associated Protein 2; VDAC1: Voltage-Dependent Anion Channel Protein 1; ACON: Aconitate Hydratase, Mitochondrial; AD: Alzheimer's Disease

Introduction

Alcohol abuse is a serious health problem worldwide, which has severe detrimental effects on the structure and function of many organs, including the brain [1]. Cerebellar degeneration is the most common effect of alcoholism [2,3]. The findings of recent clinical studies indicate that up to two thirds of chronic alcoholics exhibit signs of cerebellar degeneration [4]. Moreover, approximately 50% of alcoholic's exhibit cerebellar degeneration without clinical signs, according to a large-scale postmortem study conducted in Japan [5]. The Purkinje cells are the main targets of alcohol-induced cerebellar degeneration [6,7]. Thus, the loss and/or atrophy of Purkinje cells and reductions in the dendrite volume in the molecular layer may contribute to cerebellar atrophy [5,8].

Malnutrition, mainly in the form of thiamine deficiency, is also considered as a contributing factor to ethanol-induced neuropathological changes in the cerebellum [9,10]. However, 33% of chronic alcoholics without thiamine deficiency exhibited cerebellar atrophy according to an imaging study [11], and significant decreases in the size and density of Purkinje cells were also found in chronic alcoholics with no clinical signs of Wernicke's encephalopathy [6].

Proteomic analysis of the human brain has been employed in postmortem studies of chronic alcoholism [12,13], but the results were complicated by multifactorial effects on the human brain.

A rat model is suitable for the study of alcoholism. Alcohol exposure (dose and patterns) can be easily controlled to facilitate direct examination of the effects of ethanol on the central nervous system. In this study, we assessed changes in the protein expression profiles in the cerebellum of rats after chronic exposure to alcohol compared with controls using the Lieber–DeCarli model because it is reliable for effectively controlling the nutritional status [14]. Furthermore, this model has been used to study the brain damage caused by chronic alcohol consumption [15-17].

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Materials and Methods

Animal treatment

Eight 4-week-old male Sprague-Dawley rats weighing 140-150 g were purchased from Charles River Japan (Yokohama, Japan) and housed in individual cages in the same room at the laboratory animal facility of the Chiba University Graduate School of Medicine under a 12/12 h light/dark cycle at a constant temperature of 24°C ± 1°C. A total of eight rats were pair-fed with a nutritionally adequate liquid diet containing ethanol as 36% of the total caloric content (5% ethanol dose) or an isocaloric control diet (Oriental Yeast Co., Tokyo, Japan) for 8 weeks, as described by Lieber and DeCarli [14]. The pair-feeding method used in this study was originally designed for studies of effects of chronic alcohol consumption on the liver. All of the experiments were performed in accordance with the Animal Experimentation Guidelines of Chiba University and the study protocol was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

Tissue preparation

At the end of the ethanol exposure period, all of the rats were sacrificed separately using decapitation under light ether anesthesia. The brains were removed and the cerebellum, hippocampus, frontal cortex, striatum, amygdala, and hypothalamus were then dissected separately, cut into small pieces, and stored at −80°C until further processing.

Protein extraction

Frozen tissues (approximately 50 mg) were homogenized in 20 volumes of buffer [7 M urea, 2 M thiourea, 2% (w/v) 3-(3-cholamidopropyl) dimethylammoniumpropane sulfonate, 0.1 M dithiothreitol, 2.5% pharmalyte (pH 3–10), and protease inhibitors (Complete Mini EDTA-free; Roche Diagnostics Deutschland GmbH, Mannheim, Germany)]. The homogenates were centrifuged at 112,000 × *g* (Optima TLX Ultracentrifuge; Beckman Coulter Inc., Brea, CA, USA) at 4°C for 60 min, and the clear supernatants were transferred to new microcentrifuge tubes. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and all of the samples were frozen separately at −80°C before further processing.

Two-dimensional agarose gel electrophoresis (2-DE)

The cerebellum samples were subjected to isoelectric focusing (IEF) analysis. Agarose gels were used for the first dimension of IEF, as described previously by Oh-Ishi et al. [18]. For the second dimension, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, as described by Laemmli et al. [19]. Brain protein extracts from four pairs of rats in the 8-week treatment and control groups were analyzed on the gradient gels (a 10-20% polyacrylamide gel). Briefly, samples weighing 400 μg were subjected to IEF for the first dimension at 12,000 V and 4°C, before they were fixed in 10% trichloroacetic acid and 5% sulfosalicylic acid for 45 min at room temperature. After washing with deionized water for 60 min, the agarose gels were transferred to a 10–20% polyacrylamide gradient gel for electrophoresis in the second dimension at a constant current of 40 mA for approximately 60 min, followed by 65 mA for 180 min. The gel was fixed overnight in a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid, stained with Coomassie brilliant blue (CBB), and then destained with 30% (v/v) methanol and 10% (v/v) acetic acid.

2-DE gel image analysis

All of the 2-DE agarose gels were scanned using an Epson ES-10000G scanner equipped with a transparency adapter (Seiko Epson Corp., Suwa, Japan). Images were acquired and processed using Progenesis SameSpots image analysis software (version 3.3; Nonlinear Dynamics, Durham, NC, USA) to determine the integrated densities of the protein spots on the same 2-DE agarose gel. The statistical significance of the observed differences in each spot was assessed by Student's t-test.

In-gel protein digestion

The proteins separated by 2-DE were identified by in-gel tryptic digestion, followed by mass spectrometry (MS). The protein spots were fragmented into peptides as described by Satoh et al. [20]. Briefly, each of the protein spots was individually excised from the CBB-stained gel in approximately 1 mm squares and destained in 50% (v/v) acetonitrile (ACN) containing 50 mM NH_4HCO_3. The gel pieces were dehydrated in 100% ACN for 15 min and then dried using a TOMY CC-105 microcentrifugal vacuum concentrator (Tomy Seiko Co. Ltd, Tokyo, Japan) for at least 60 min. The gel pieces were rehydrated and immersed in 10–30 μL of 25 mM NH_4HCO_3 containing 50 ng/μL of proteomic-grade trypsin (Roche Diagnostics Deutschland GmbH) for 45 min at 4°C. After removing the unabsorbed solution, the gel pieces were incubated in 10–20 μL of 25 mM NH_4HCO_3 buffer for 24 ± 2 h. The solution containing digested protein fragments was transferred into a new tube and stored at 4°C. The peptide fragments remaining in the gel were extracted in a minimal volume of 5% (v/v) formic acid containing 50% (v/v) ACN for 20 min at room temperature.

MS analysis and database search

The digested peptides were desalted and selectively enriched with C18-StageTips [21]. Next, the enriched samples were injected into a trap column (C18, 0.3 × 5 mm; Dionex Corporation, Sunnyvale, CA, USA) and an analytical column (C18, 0.075 × 120 mm; Nikkyo Technos Co. Ltd, Tokyo, Japan), which were attached to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex Corporation). The flow rate of the mobile phase was 300 nL/min. The solvent composition of the mobile phase was programmed to change over a 120 min cycle with variable mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) relative to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–10% B for 5 min, 10–13.5% B for 35 min, 13.5–35% B for 65 min, 35–90% B for 4 min, 90% B for 0.5 min, 90–5% B for 0.5 min, and 5% B for 10 min. Purified peptides were transferred from the HPLC system to a LTQ-Orbitrap XL hybrid ion-trap Fourier transform mass spectrometer (Thermo Scientific, San Jose, CA, USA). The Mascot search engine (version 2.2.6; Matrix Science Ltd., London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the UniProtKB Rattus database (SwissProt 2011x, August 2011, 7645 entries), where the database search parameters were as follows: Peptide mass tolerance=2 ppm, fragment tolerance=0.6 Da, enzyme=trypsin, up to one missed cleavage was allowed, and variable modifications=methionine oxidation. The minimum criterion for protein identification was set as a false discovery rate of <1%, which was estimated by searching against a randomized decoy database created by the Mascot Perl program supplied by Matrix Science Ltd.

Western blotting

Western blotting analysis of the proteins identified by 2-DE was performed to confirm the differences in the protein expression levels in

cerebellum between the alcohol-fed and pair-fed control groups. Same amount of protein extracts (5 μg per lane) were separated by SDS-PAGE on 10–20% gradient gels or a 7.5% gel (XV PANTERA Gel; DRC, Tokyo, Japan) for 18 min at 240 V, and then transferred onto polyvinylidene fluoride membranes (EMD Millipore Corporation, Bedford, MA, USA) in a tank-transfer apparatus (Bio-Rad Laboratories, Inc.) at 10 V overnight (17 h). The membrane was blocked with 0.5% low-fat skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated with the following primary antibodies (all from Abcam plc., Tokyo, Japan) in 0.5% low-fat milk in TBS-T: (a) Chicken anti-rat microtubule-associated protein 2 (MAP2) antibody (ab5392; dilution, 1:100,000), (b) Mouse anti-rat voltage-dependent anion channel 1 (VDAC1) antibody (ab14734; dilution, 1:10,000), (c) Rabbit anti-rat aconitate hydratase mitochondrial (ACON) antibody (ab129105; dilution, 1:50,000), and (d) Mouse anti-rat β-actin antibody (ab49846; dilution, 1:1,000,000). The blots were washed three times with TBS-T, each for 5 min and then incubated with the following secondary antibodies in blocking buffer for 1 h: (a) Anti-chicken antibody (dilution, 1:10,000; GenWay Biotech, Inc., San Diego, CA, USA), (b) Anti-mouse antibody (dilution, 1:1000; Dako Denmark A/S, Glostrup, Denmark), and (c) Anti-rabbit antibody (dilution, 1:5000; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Antigens bound to the membrane were detected using ECL plus enhanced chemiluminescence detection reagents (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK), and the band intensities were quantified using LPR-400EX chemiluminescence imager imaging analysis software (Taitec, Tokyo, Japan).

Statistical analysis

All of the numerical data were expressed as means ± standard deviations. The statistical significance of differences was assessed using paired *t*-tests. $p<0.05$ was considered statistically significant.

Results

Animal data

Before feeding for 8 weeks, the Sprague-Dawley rats weighed 140–150 g. At the end of feeding, the weights of the control and alcohol-fed rats were 357 ± 19 g and 362 ± 29 g, respectively. The total caloric intake of the control and alcohol-fed rats was 3995 ± 289 kcal and 3993 ± 291 kcal, respectively. There were no significant differences in body weight or caloric intake between the alcohol-fed groups and pair-fed controls during the period of alcohol exposure. There were no significant cerebellar symptoms, such as ataxic gate or shaking, in the alcohol-fed rats. All of the alcohol-fed rats had alcohol hepatic steatosis (Supplementary Figure 1).

Identification of proteins with significantly changed expression profiles after alcohol exposure

A representative 2-DE pattern of the protein extract from a control rat is shown in Figure 1A. In total, 615 protein spots were detected in each of the eight gels. Protein spots with significant changes in intensity (Student's t-test; $p<0.05$) ≥1.4-fold are shown in Figure 1B. MAP2, ACON, and VDAC1 were identified in spots 1, 2, and 3, respectively; by MS analysis and database search (Table 1). MAP2 is known as a major cytoskeletal protein, ACON as an enzyme that catalyzes the isomerization of citrate to isocitrate via cis-aconitate in mitochondria, and VDAC1 as a channel at the outer mitochondrial membrane.

Validation of differentially expressed proteins in cerebellar tissues and other brain region tissues in control and alcohol-fed rats

Western blotting indicated MAP2 expression was significantly lower (control: 0.3 ± 0.08; alcohol: 0.18 ± 0.03; $p=0.0396$) and VDAC1 expression was significantly higher (control: 0.67 ± 0.26; alcohol: 0.73 ± 0.26; $p=0.0264$) in the cerebellums of alcohol-fed rats compared with the pair-fed controls (Figure 2). However, there were no significant differences in the ACON expression levels between the two groups (Figure 2). Western blotting was performed for MAP2 and VDAC1 in other brain regions, including the hippocampus, frontal cortex, striatum, amygdala, and hypothalamus, for the pair-fed control and alcohol-fed groups, but there was no significant change in the protein expression levels of MAP2 and VDAC1 in the alcohol-fed and pair-fed control groups (Supplementary Figure 2).

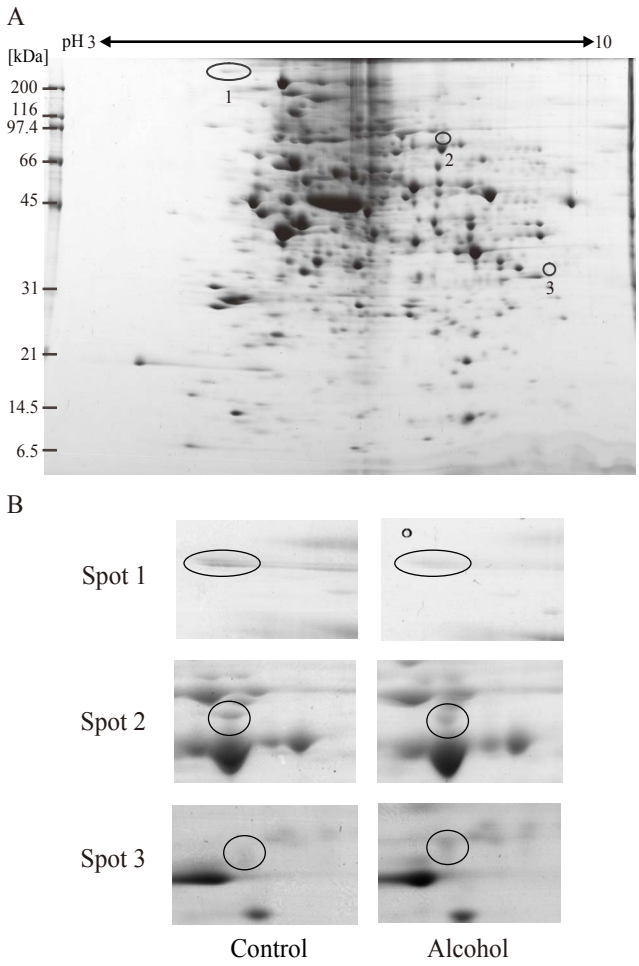

Figure 1: Representative 2-DE agarose gel CBB staining pattern for the proteins expressed in the cerebellum of pair-fed controls (A). The spot densities in four pairs of an alcohol-fed rat and a pair-fed control were compared using Student's t-test. Magnified 2-DE maps of selected spots from CBB staining (B).

Discussion

The cerebellum is one of the regions that are most susceptible to alcohol-induced brain damage [3], but the underlying mechanisms of cerebellar degeneration following chronic alcohol exposure remain unclear.

In this study, we compared the protein expression profiles in the cerebellar tissues of control rats and chronically alcohol-fed rats based on agarose 2-DE proteomics followed by immunoblotting analysis. We employed agarose gels in the first dimension of 2-DE proteomics. This method is useful for resolving high molecular mass proteins (>150

Protein spot no.[a]	Fold change[b]	Protein identification					
		Protein name	Experimental MW (Da)	Theoretical MW(Da)[c]	Matched peptides	MS/MS[d]	Mowse score[e]
1	−1.6	Microtubule-associated protein 2	280,000	202,288	110	78	3316
2	−1.4	Aconitate hydratase, mitochondrial	85,000	85,380	10	10	294
3	1.5	Voltage-dependent anion channel protein1	36,000	30,737	12	9	415

[a]Protein spots were described in Figure 1.
[b]Intensity of spots in 2-DE gels across the 8 matched samples was measured using the Progenesis Samespot software.
[c]Theoretical molecular mass (Da) based on NCBI BLAST database.
[d]The number of unique peptides of MS/MS. The minimum criterion was set as an FDR of less than 1%.
[e]Mowse scores of candidate proteins.

Table 1: Differentially expressed proteins in the cerebellum of chronically alcohol-fed rats compared with controls.

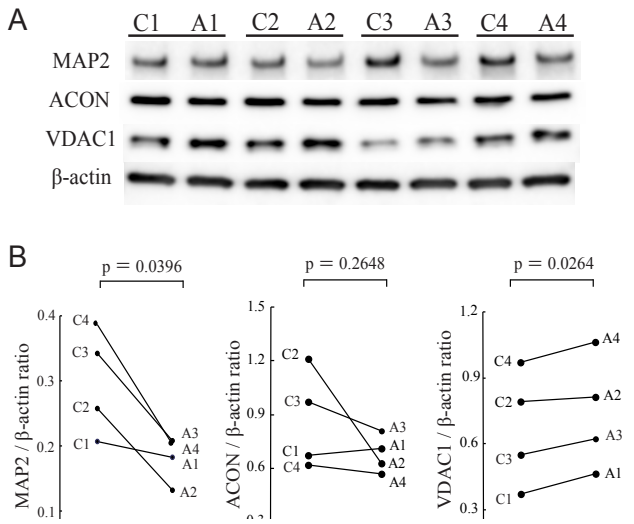

Figure 2: Immunoblot analysis of MAP2, ACON, and VDCA1 in the cerebellum of control rats (N=4) and those fed the Lieber–DeCarli diet for 8 weeks (N=4) (A). The intensity of each band was determined by imaging analysis and the relative protein levels in tissues were calculated, where the levels were normalized against β-actin (B). A dot represented the levels of each sample, and the dots of an alcohol-fed rat and a pair-fed control were connected with a straight line. Each pair was compared using paired t-test, and the band volume of MAP2 was significantly lower, whereas that of VDAC1 was significantly higher. No significant changes were observed in the intensity of the ACON band (C: Control rat; A: Alcohol-fed rat).

kDa), which are difficult to resolve with the conventional immobilized pH gradient 2-DE method [18,22]. We identified a high molecular weight protein (280 kDa), MAP2, which is a major cytoskeletal protein in neuron dendrites and in the cell body, where it is indispensable for microtubule stability and synaptic plasticity [23]. The dendrites of neurons in the hippocampus of alcohol-exposed rats were fragmented with abnormal structures [24]. The low expression level of MAP2 following chronic alcohol exposure has been detected previously in the hippocampus by immunostaining methods using both *in vitro* and *in vivo* studies [24,25]. However, the lower expression of MAP2 in cerebellar tissues after chronic alcohol consumption has not been shown previously. In addition, low expression levels of MAP2 have been observed in other excitotoxicity-related neurodegenerative disorders [26], and excitotoxicity is a major molecular mechanism that contributes to chronic alcohol-induced cerebellar degeneration neuropathology [2,27]. Therefore, alcohol-induced excitotoxicity may induce the low expression levels of MAP2 following long-term alcohol exposure, which may indicate the changes in cerebellar neuronal cytoskeleton.

In the present study, we also demonstrated that VDAC1 was overexpressed in the cerebellum of alcohol-fed rats. VDAC1, which is known as mitochondrial porin, is located in the mitochondrial outer membrane where it is responsible for ATP transport and calcium homeostasis in various cells [28]. VDAC1 is also regarded as a modulator of mitochondrial-mediated apoptotic cell death [28,29]. Mitochondria are the main producers of reactive oxygen species as well as the major targets of oxidative stress [30]. Moreover, many studies have reported disruption of the properties of the mitochondrial membrane in the brain by alcohol-induced oxidative stress [31]. The apoptosis of Purkinje and granular cells in the cerebellum of adult rats with chronic ethanol treatment has been described previously [32], but the underlying mechanisms are still unknown. The overexpression of VDAC1 has been reported in the accumbens nucleus of rats with chronic alcohol exposure [33]. In the present study, we detected the overexpression of VDAC1 in the cerebellum of rats after chronic alcohol treatment. Thus, the overexpression of VDAC1 in the brain might indicate apoptotic cell death due to alcohol-induced oxidative stress.

In addition, VDAC1 overexpression has also been demonstrated in other neurodegenerative disorders by proteomic analysis, such as Alzheimer's disease (AD) [34]. Reduced VDAC1 expression was shown to have a protective role in the brain tissue of animal models of AD [35], but further research by overexpression/inhibition techniques is needed to determine whether the inhibition of VDAC1 can prevent oxidative stress and apoptosis in the cerebellum with long-term alcohol exposure.

In this study, in chronically alcohol-fed rats, we observed no significant changes in the expression levels of MAP2 and VDAC1 in other brain regions, including the hippocampus, frontal cortex, striatum, amygdala, and hypothalamus (Supplementary Figure 2). These results support previous findings that the cerebellum is more susceptible to the detrimental effects of alcohol than other brain regions [3]. Vermis is known as the most alcohol-sensitive area in cerebellum [7]. Changes in the expression of these proteins could be related to the protein profile alterations in the vermis. In this study, we found there were no changes in the expression levels of both MAP2 and VDAC1 in one of the main alcohol-sensitive regions, the hippocampus (Supplementary Figure 2). Several studies showed low expression levels of MAP2 in hippocampus after chronic alcohol exposure, but they used embryonic rats or adult rats with high amount of alcohol exposure [24,25]. Previous studies have reported no loss of neurons in the hippocampus of alcoholics [36]; thus, these results suggest hippocampal neurogenesis effects may help to compensate for the detrimental effects of ethanol on neurons.

The rats used in our study were exposed to ethanol between 4-12 weeks of age, their adolescent and adult stages. The ethanol effects to the adolescent rat cerebellum might be different from those to the adult, and the results in our study should be carefully considered.

We could not find the cerebellar symptoms in alcohol-fed rats. However, it is possible that longer alcohol feeding would lead the

cerebellar damages. The alterations in MAP2 and VDAC1 expressions found in this study could be the early changes in the alcohol-induced cerebellum degeneration.

Conclusion

In conclusion, we identified changes in the expression levels of MAP2 and VDAC1 by 2-DE in the cerebellum of rats subjected to an 8-week ethanol exposure regimen using the Lieber–DeCarli liquid diet. The low expression of MAP2 and overexpression of VDAC1 in the cerebellum of rats exposed chronically to alcohol in this study may provide insights into the mechanisms of neuronal cytoskeleton alterations and neuronal apoptotic changes in the cerebellum following chronic alcohol exposure.

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