Amino Acids (2012) 43:1705–1711
DOI 10.1007/s00726-012-1254-6

ORIGINAL ARTICLE

Taurine protects cerebellar neurons of the external granular layer against ethanol-induced apoptosis in 7-day-old mice

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Received: 1 November 2011 / Accepted: 14 February 2012 / Published online: 2 March 2012

Abstract Acute alcohol administration is harmful especially for the developing nervous system, where it induces massive apoptotic neurodegeneration leading to alcohol-related disorders of newborn infants. Neuroprotection against ethanol-induced apoptosis may save neurons and reduce the consequences of maternal alcohol consumption. Previously we have shown that taurine protects immature cerebellar neurons in the internal granular layer of cerebellum from ethanol-induced apoptosis. Now we describe a similar protective action for taurine in the external layer of cerebellum of 7-day-old mice. The mice were divided into three groups: ethanol-treated, ethanol + taurine-treated and controls. Ethanol (20% solution) was administered subcutaneously at a total dose of 5 g/kg (2.5 g/kg at time 0 h and 2.5 g/kg at 2 h) to the ethanol and ethanol + taurine groups. The ethanol + taurine group also received subcutaneously two injections of taurine (1 g/kg each, 1 h before the first dose of ethanol and 1 h after the second dose of ethanol). To verify apoptosis, immunostaining for activated caspase-3 and TUNEL staining were made in the mid-sagittal sections containing lobules I–X of the cerebellar vermis at 8 h after the first ethanol injection. Ethanol induced apoptosis in the cerebellar external granular layer. Taurine treatment significantly reduced the number of activated caspase-3-immunoreactive and TUNEL-positive cells. Taurine has thus a neuroprotective antiapoptotic action in the external granular layer of the cerebellum, preserving a number of neurons from ethanol-induced apoptosis.

Keywords Taurine neuroprotection · Ethanol-induced apoptosis · TUNEL staining · Activated caspase-3 · External granular layer · Developing cerebellum

Abbreviations

ANOVA	Analysis of variance
EGL	External granular layer
FAS	Fetal alcohol syndrome
IGL	Internal granular layer
IR	Immunoreactive
P0	Day of birth
P6	Postnatal day 6
P7	Postnatal day 7
P8	Postnatal day 8
P21	Postnatal day 21
PBS	Phosphate buffered saline
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

Introduction

It is well established that alcohol consumption during pregnancy is harmful to fetus, especially to its developing nervous system (Warren and Bast 1988; Dalen et al. 2009). However,

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in the United States about 11% of pregnant women continue to consume alcohol during their pregnancies (The NSDUH Report 2008). Depending on the frequency of drinking, the amount of alcohol consumed and the period of pregnancy, spontaneous abortion (Kline et al. 1980) or preterm birth (Parazzini et al. 2003) may result or newborn children may suffer from the fetal alcohol syndrome (FAS) which includes growth retardation, structural brain abnormalities, behavior and cognitive problems, and learning difficulties, for instance (Chudley et al. 2005). The prevalence of children affected by the prenatal alcohol exposure (FAS and alcohol-related birth defects) remains too high, being about one percent of all children born in the United States, even though not all babies born from alcohol-drinking women have symptoms of alcohol-related disorders (May and Gossage 2001). In rodents in vivo (Ikonomidou et al. 2000; Dikranian et al. 2005) and humans in vitro (Hao et al. 2003) alcohol induces massive death of neurons in the developing brain by apoptosis (Hotchkiss et al. 2009). Such a widespread apoptosis may account for the morphological and functional disorders and retardation in learning of children with FAS (Hao et al. 2003). Apoptosis is a complex, tightly regulated process which occurs stepwise via a consecutive activation of specific proteins which destroy the cell (Hotchkiss et al. 2009). The possibility of interfering with this process with drugs to prevent ethanol-induced apoptosis could save a large number of neurons. The amino acid taurine is a good candidate for achieving this goal since it is normally present at high concentrations in brain tissue (Huxtable 1992; Sturman 1993), has no side-effects even if administered at high doses (Airaksinen et al. 1980), and is involved in apoptosis regulation (Takatani et al. 2004; Wu et al. 2009). Recently, we demonstrated that taurine can protect neurons against ethanol-induced apoptosis in glutamatergic excitatory granule neurons in the internal granular layer (IGL) of the cerebellum in 7-day-old mice (Taranukhin et al. 2009), but not in inhibitory GABAergic Purkinje cells in 4-day-old mice (Taranukhin et al. 2010). We have continued our investigation on the possible neuroprotective role of taurine against ethanol-induced apoptosis and focused our attention on another neuronal population, the external granular neurons of the cerebellum, to verify whether the taurine effect is limited to glutamatergic neurons such as granule cells.

Materials and methods

Animals and experimental protocols

Adult NMRI mice for breeding were purchased from Harlan, Netherlands. Seven-day-old infant male mice were used in the experiments (day of birth is day 0). The experiments on animals were carried out in accordance with the European Community Council Directive 86/609/EEC. All efforts were made to minimize the suffering of the animals.

In the first set of experiments 7-day-old infant male mice from P4 to P6 were injected five times with 1 g/kg taurine with 12 h intervals to study whether taurine alone could have any effect on apoptosis of granule cells in the external cerebellar granule cell layer. In the further experiments the possible influence of individual hereditary characters on experimental results was reduced by dividing the mice in each litter into three groups: ethanol-treated, ethanol + taurine-treated and controls. Apoptosis in the developing cerebellum was induced by an acute alcohol administration to pups (Ikonomidou et al. 2000; Dikranian et al. 2005; Taranukhin et al. 2009, 2010). In brief, 20% w/v ethanol solution in sterile saline was administered subcutaneously to the ethanol and ethanol + taurine groups at a total dose 5 g/kg (2.5 g/kg at 0 h and 2.5 g/kg again at 2 h). These injections elevated the concentration of ethanol in blood at least to 40 mmol/l for 8 h (Ikonomidou et al. 2000; Dikranian et al. 2005). Taurine administration did not affect the concentration of ethanol in blood (data not shown). Taurine diluted in saline was injected subcutaneously to the ethanol + taurine group at total dose 2 g/kg (1 g/kg of taurine 1 h before the first dose of ethanol and 1 g/kg of taurine 1 h after the second dose of ethanol). This taurine dose and regimen of injections have been tested in our previous studies (Taranukhin et al. 2009, 2010) and proven to have protective effects on neurons in the IGL of the developing cerebellum. This taurine treatment elevated the concentration of taurine in blood from 1 to 13 mmol/l and maintained it at a high level (above 10 mmol/l) during the whole 9-h experiment (Taranukhin et al. 2010). However, the taurine concentrations in the whole brain (622.5 ± 63.8 mmol/kg protein, mean ± SD) and in the whole cerebellum (616.3 ± 89.1 mmol/kg protein, mean ± SD) were not significantly altered. Instead of the taurine injections the ethanol group received two saline injections in the equal volume. The control group received saline injections equal in volume and regimen to those given to the ethanol + taurine group.

Eight hours after the first ethanol injection the mice were killed by decapitation. The cerebella were rapidly excised and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 3 days at 4°C. After a routine histological processing, the cerebella were embedded in paraffin and cut into 5-μm thick mid-sagittal sections containing lobules I–X of the cerebellar vermis (Fig. 1).

Immunohistochemistry

To reveal neurons with activated caspase-3 in the cerebellum, immunohistochemistry was performed using polyclonal activated caspase-3 antibody [cleaved caspase-3 (Asp 175) antibody, Cell Signaling Technology Inc.] (Taranukhin

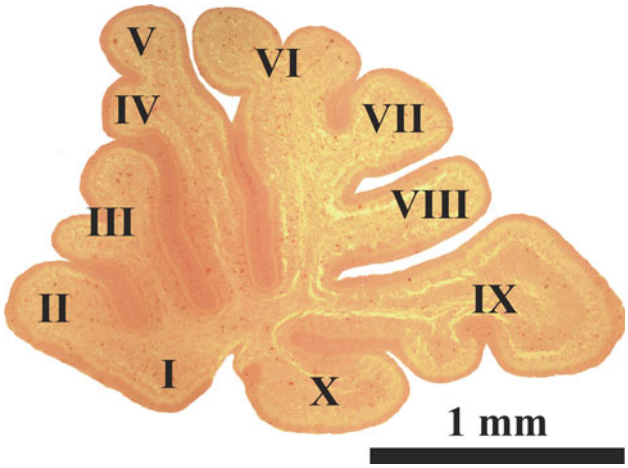

Fig. 1 Mid-sagittal section of the cerebellum showing an overview of the cerebellar vermis from the 7-day-old mouse. The section was obtained from a mouse in the ethanol-treated group 8 h after the first ethanol administration. The section is stained immunohistochemically with antibodies to activated caspase-3 and with hematoxylin-eosin counterstaining to enhance histological details. The lobules of the cerebellar vermis are marked I–X. Magnification ×75. *Scale bar*: 1 mm

et al. 2010). The cerebellar sections were deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. After antigen retrieval by microwave [20 min at 1,000 W in 0.01 M citrate buffer (pH 6.0)], washing in PBS and blocking with 0.5% hydrogen peroxide in PBS for 20 min, the specimens were preincubated for 30 min in serum-blocking solution (1% bovine serum albumin and 0.3% Triton X-100 in PBS). Thereafter, the specimens were incubated with primary antibody to activated caspase-3 (diluted 1:200 in serum-blocking solution) in moist chambers overnight at 4°C. The next day, sections were washed PBS and incubated for 1 h with biotinylated secondary antibody (goat anti-rabbit 1:500 in blocking solution) and ABC complex (30 min) (Vectastain Elite ABC Kit, Vector Laboratories, Inc.). Diaminobenzidine was used as a chromogen to visualize the sites expressing activated caspase-3 immunoreactivity. The sections serving as negative controls were incubated without the primary antibody to rule out nonspecific staining. Finally, all sections were counterstained with hematoxylin-eosin to enhance histological details, dehydrated and mounted.

Detection of cell death in situ

DNA fragmentation is one of the wide accepted markers by which apoptotic cells are recognized. To detect DNA fragmentation of cell nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction was applied to the paraffin sections using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Germany). After deparaffinization, the sections were irradiated with microwaves in 0.01 M citric acid buffer (pH 6.0) for 10 min at 750 W. No inhibition of endogenous peroxidase was performed because H_2O_2 weakens terminal deoxynucleotidyl transferase activity (Migheli et al. 1995) and induces DNA breaks (Wijsman et al. 1993). Sections were incubated with the TUNEL reaction mixture for 60 min at 37°C. Further incubation with peroxidase-conjugated antibody was performed for 30 min at 37°C. The sections were stained with diaminobenzidine for 10 min at the room temperature and then counterstained with hematoxylin–eosin.

Microscopy, image analysis and cell counting

The sections were processed under standardized conditions for immunohistochemistry or TUNEL assay to minimize variability in labeling conditions. Further, the sections were studied with light microscopy (magnifications ×75 and ×750) using an image analysis system comprising of an IBM PC, Nikon Microphot-FXA microscope, SensiCam digital camera (PCO Computer Optics GmbH), Image-Pro Plus (Media Cybernetics) to analyze caspase-3 immunoreactivity and TUNEL-staining. At least five sections cut at the same level of the cerebellar vermis from every animal were analyzed. The number of neurons from the external granular layer (EGL) (Fig. 2) labeled for caspase-3 activation or TUNEL assay was counted in every slice and the area of the EGL in each lobule was measured. The data are presented as the average number of labeled cells in the EGL per mm^2 for each experimental group.

Data expression and statistical analysis

One-way analysis of variance (ANOVA) was used to compare the number of activated caspase-3-immunoreactive (IR) cells and TUNEL-positive cells among the experimental groups. When ANOVA showed a significant difference, the post-hoc Bonferroni test was applied to demonstrate the difference. Each value is expressed as mean ± standard deviation. Differences were considered significant when the calculated P value was at least <0.01.

Results

Effects of taurine on the number of activated caspase-3-IR neurons

Activated caspase-3-IR cells in the EGL were found in each cerebellar lobule in the 7-day-old saline-treated control mice. The repeated administrations of taurine alone

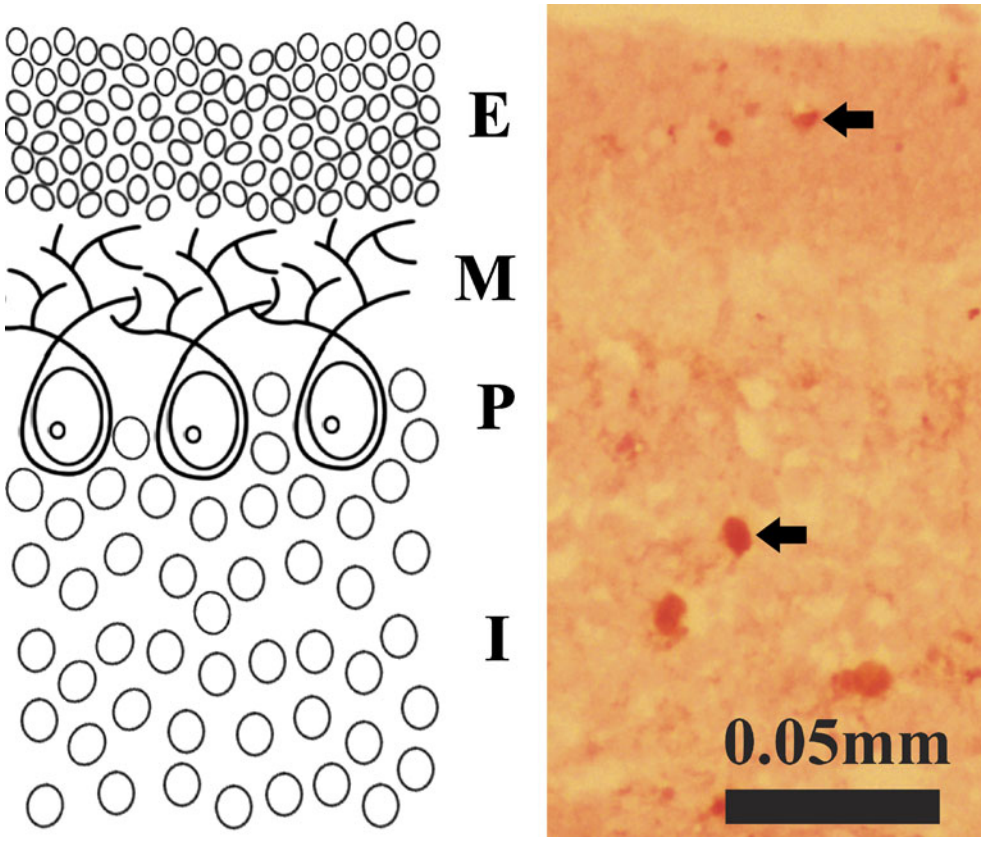

Fig. 2 Scheme and microscopic picture illustrating the layers of the cerebellum in the 7-day-old mouse. Microscopic picture presents a part of lobule III stained immunohistochemically with antibodies to activated caspase-3 and with hematoxylin–eosin counterstaining to enhance histological details. The section was obtained from a mouse in the ethanol-treated group 8 h after the first ethanol administration. The layers of the cerebellar lobule are marked as follows: *E* external granular cell layer, *M* molecular layer, *P* Purkinje cell layer, *I* internal granular cell layer. The *arrows* indicate examples of caspase-3-IR cells in the external and internal granular layers. Magnification ×750. *Scale bar*: 0.05 mm

had no effect on the number of caspase-3-IR neurons in any cerebellar lobule (data not shown). Ethanol administration increased the number of activated caspase-3-IR neurons 1.5–3.5 times in all lobules, the effect being statistically significant ($P < 0.01$) in lobules I–II, III, VII, VIII and X (Fig. 3). However, taurine decreased in the ethanol-treated mice the number of activated caspase-3-IR cells in the EGL in each lobule. This effect was statistically significant ($P < 0.01$) in lobules I–II, III, IV–V, VIII and X, from 35 to 53% of the neurons affected by ethanol being saved.

Effects of taurine on the number of TUNEL-positive cells

The administrations of taurine alone did not either affect the number of TUNEL-positive cells in the EGL in the cerebellum (data not shown). A small amount of cells with fragmented DNA labeled by the TUNEL assay were detected in the EGL of 7-day-old mice in the saline-treated control group showing physiological cell death during normal development. The number of TUNEL-positive cells in the EGL after the ethanol treatment was markedly increased ($P < 0.001$) in all lobules, being 3.0–6.5 times greater than in the control group (Fig. 4). Taurine treatment protected the EGL cells from DNA fragmentation. This effect was statistically significant ($P < 0.01$) in lobules III, IV–V, VII, VIII, IX and X, varying from 43 to 69% compared to the ethanol group.

Discussion

The EGL of the cerebellum is a temporary structure which disappears when the cerebellum becomes mature (Fujita 1967). In mice, the EGL forms by the embryonic day 17.5, when granular cells intensively proliferate and starting at P6 they begin to migrate to the IGL. By P21 the migration and maturation of granular cells are complete and the EGL disappears (Espinosa and Luo 2008). Neuronal apoptosis in the EGL in mice is observed already at P0 and it increases with a parabolic pattern to peak at P8 (Cheng et al. 2011). It reflects the rate of normal physiological cell death

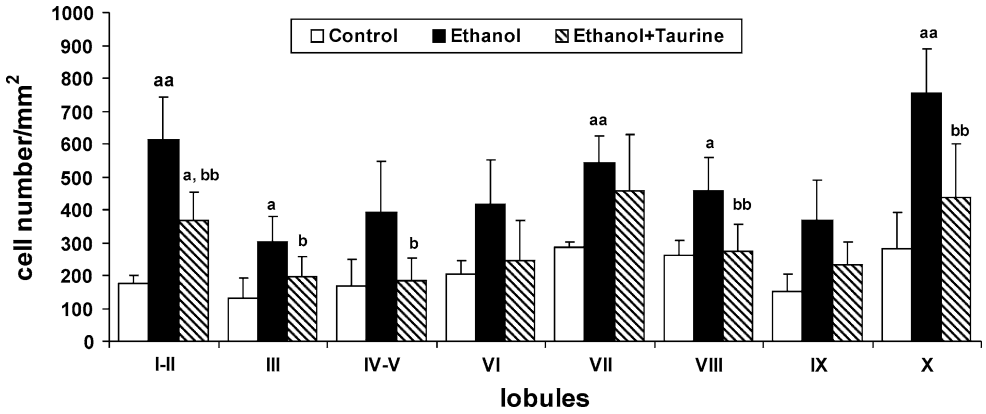

Fig. 3 Effect of ethanol and ethanol + taurine treatment on the number of activated caspase-3-immunoreactive neurons in the external granular layer of the cerebellar vermis of the 7-day-old mice. Number of activated caspase-3-IR cells in the control group (*open bars*), ethanol-treated group (*filled bars*) and ethanol + taurine-treated group (*hatched bars*). The results are given per mm^2 with standard deviations. Number of animals in each group is five and the number of sections analyzed from each animal 5–8. The significance of differences compared to the control group: $^{a}P < 0.01$; $^{aa}P < 0.001$. The significance of differences between the ethanol and ethanol + taurine groups: $^{b}P < 0.01$; $^{bb}P < 0.001$

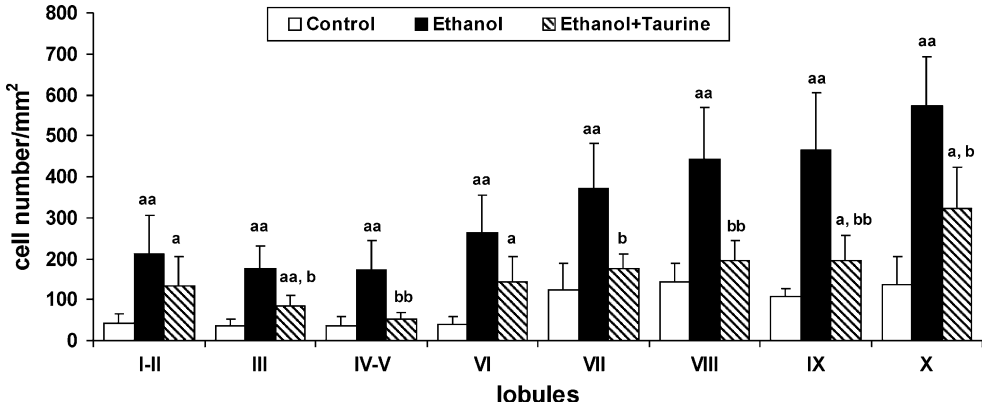

Fig. 4 Effect of ethanol and ethanol + taurine treatment on the number of TUNEL-positive cells in the external granular layer of the cerebellar vermis of 7-day-old mice. Number of TUNEL-positive cells in the control group (*open bars*), ethanol-treated group (*filled bars*) and ethanol + taurine-treated group (*hatched bars*). The results are given per mm^2 with standard deviations. Number of animals in each group is five and the number of sections analyzed from each animal 5–8. The significance of differences compared to control group: $^{a}P < 0.01$; $^{aa}P < 0.001$. The significance of differences between the ethanol and ethanol + taurine groups: $^{b}P < 0.01$; $^{bb}P < 0.001$

occurring at this period of development. In our experiments apoptotic cells labeled as activated caspase-3-IR and TUNEL positive cells were also discernible in the EGL of all studied cerebellar lobules from the control group in 7-day-old mice.

It is known that the developing brain is extremely sensitive to the adverse effects of alcohol during the period of synaptogenesis, also known as the brain growth spurt, the timeframe of which varies in different species. It is the last trimester of pregnancy in humans and the first postnatal week in mice (Rice and Barone 2000). Ethanol administration to 7-day-old mice induced widespread neuronal degeneration in the brain and cerebellum exhibiting the biochemical and ultrastructural alterations indicative of apoptotic cell death such as activation of caspase-3, cleavage of DNA, and chromosomal, and morphological changes (Dikranian et al. 2005). In our experiments ethanol administration to 7-day-old mice increased apoptosis in the EGL of all cerebellar lobules as indicated by the greater number of activated caspase-3-IR and TUNEL-positive neurons in the ethanol-treated group compared to the control group.

Taurine is essential for normal development and present at high concentrations during the early ontogenesis (Oja and Piha 1966; Oja et al. 1968; Sturman 1993). It has many physiological functions (Huxtable 1992; Oja and Saransaari 2007) and protects many types of cells from different damaging effects such as ischemia (Takatani et al. 2004; Taranukhin et al. 2008), high glucose level (Ulrich-Merzenich et al. 2007), oxidative stress (Das

et al. 2009) and ethanol intoxication (Taranukhin et al. 2009, 2010). The intracellular taurine concentration is about 400 times greater than the concentration in the intercellular space (Lerma et al. 1986; Molchanova et al. 2004). As a small molecule taurine freely penetrates from capillaries into interstitial fluid. It can penetrate intracellularly into brain cells in the developing brain but less readily in the adult brain (Oja et al. 1976). Nevertheless, it was now found to reduce the number of activated caspase-3-IR cells and TUNEL-positive cells in the EGL in ethanol-treated developing mice but did not have any effect on apoptosis when administered alone. Apoptosis induced by ethanol in infant rodents is Bax-dependent and manifests itself mainly through the intrinsic mitochondrial pathway (Young et al. 2003; Nowoslawski et al. 2005), causing mitochondrial membrane disruption, cytochrome *C* release, apoptosome formation, activation of caspase-9, activation of caspase-3, fragmentation of DNA and cell death. Taurine can interrupt this apoptotic process at some of these stages. Thus, for instance, taurine decreases intracellular free Ca^{2+} and prevents the activation of calpain (calcium-dependent protease). In this manner it protects mitochondrial membranes from disruption (Wu et al. 2009).

A balance between the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 is very important for cell survival. A decrease in the Bcl-2 level in cells leads to translocation of Bax to mitochondria, disruption of mitochondrial membranes and the release of cytochrome *C* from the mitochondria to the cytosol (Hagberg et al. 2009). The treatment with taurine has been found to restore the pool of Bcl-2 and protect cells against apoptosis (Wu et al. 2009). Taurine may also be able to rescue cells from apoptosis after the release of cytochrome *C* from mitochondria. For example, in ischemic cardiomyocytes taurine suppresses the formation of the Apaf-1/caspase-9 apoptosome, prevents caspase-9 activation and thereby preserves cells from apoptosis (Takatani et al. 2004). While some or all of these mechanisms may account for the protection of EGL neurons by taurine against ethanol-induced apoptosis, the protection is incomplete since only about one half of the dying EGL neurons survived. The similar results we had found earlier on the cerebellar IGL neurons of 7-day-old mice (Taranukhin et al. 2009, 2010), where taurine also saved about 50% of dying neurons from ethanol-induced apoptosis. The reason for this partial protection remains unclear and requires further research. In an attempt to save more neurons we increased the taurine dosage two and three-fold, but then the combination of taurine and ethanol proved to be toxic killing 50 and 100% of treated mice, respectively (Taranukhin et al. 2011).

Conclusions

We here show that the acute exposure of 7-day-old mice to ethanol causes extensive apoptosis in the EGL of the developing cerebellum. Taurine treatment has protective effects on EGL neurons, significantly alleviating ethanol-induced apoptosis. These data are in a good accordance with our previous findings on taurine protection of neurons in the IGL. However, taurine saved only a part of neurons, but did not totally abolish ethanol-induced apoptosis. The possible mechanisms of neuroprotective action of taurine likely involve regulation of intracellular Ca^{2+}, restoration of the pool of Bcl-2 and inhibition of caspase-9 activation.

Acknowledgments The authors are deeply grateful to Mrs. Ulla M. Jukarainen for excellent technical assistance. This study was supported by the competitive research funding of the Pirkanmaa Hospital District and the Finnish Foundation for Alcohol Studies.

Conflict of interests The authors declare that they have no competing interests.

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