Chapter 16
Taurine Protects Immature Cerebellar Granullar Neurons against Acute Alcohol Administration

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Abstract Acute ethanol administration causes extensive apoptosis throughout the nervous system. We studied the protective effect of taurine on alcohol-induced apoptosis in the cerebellum of developing mice. Taurine rescued a part of immature neurons by markedly reducing caspase-3 immunoreactivity and the number of TUNEL-positive cells in most cerebellar lobules.

Abbreviations *caspase-3-IR*, caspase-3-immunoreactive; *GL*, grey level

16.1 Introduction

The central nervous system is extremely sensitive to ethanol during its development and the periods of vulnerability are temporally well defined. Exposure to ethanol during the last trimester of human gestation can produce a broad spectrum of neuropathological consequences (Clarren et al. 1978 Famy et al. 1998 Spadon et al. 2007). The approximate equivalent of this period in rodents is the first postnatal week (Dobbin and Sands 1979; Rice and Barone 2000). Acute alcohol administration to mice during this period causes extensive apoptosis throughout the central nervous system (Olney et al. 2002a). Taurine has been shown to interact with the effects of ethanol (Olive et al. 2002). For instance, it modulates ethanol-stimulated locomotion (Aragón et al. 1992) and prolongs ethanol-induced sedation when given intracerebroventricularly to mice (Ferko 1987; Ferko 1988). Furthermore, ethanol administration has been shown to elicit an increase of extracellular taurine in the rat cerebral cortex and hippocampus (Dachhour and De Witte 2000). In the present work we studied the possible neuroprotective effects of taurine in ethanol-induced apoptosis in the developing mouse cerebellum. The experiments

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were performed on 7-day-old male mice. This age was chosen as the most sensitive to ethanol-induced apoptotic neurodegeneration. Moreover, spontaneous apoptosis is a common prominent phenomenon at this age (Wood et al. 1993).

16.2 Materials and Methods

16.2.1 Animals and Treatments

Seven-day-old infant male NMRI mice were used in all experiments. The animals were divided into three groups: ethanol-treated, ethanol+taurine-treated and controls. Ethanol was mixed in sterile saline to a 20% solution and administered subcutaneously at a total dose of 5 g/kg (2.5 g/kg at time zero and 2.5 g/kg again at 2 h) to the ethanol and ethanol+taurine groups. The ethanol+taurine group also received two injections of taurine (1 g/kg diluted with saline). The first taurine injection was given one hour before the first ethanol injection and the second taurine injection one hour after the second ethanol injection. The control animals were given saline subcutaneously. Eight hours after the first ethanol injection all animals were killed by decapitation. Their cerebella were rapidly excised, fixed in 4% paraformaldehyde, embedded in paraffin and cut with a microtome into 5-μm thick mid-sagittal sections containing lobules II-X of the cerebellum.

16.2.2 Immunohistochemistry

The sections were deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. After antigen retrieval by microwave (20 min at 1000 W in 0.01 M citrate buffer (pH 6.0), washing in phosphate buffered saline (PBS) and blocking with 0.5% hydrogen peroxide in PBS for 20 min, 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 polyclonal activated caspase-3 antibody [cleaved caspase-3 (Asp 175) antibody] Cell Signaling Technology Inc., diluted 1:200 in serum-blocking solution] in moist chambers overnight at 4°C. After incubation with the primary antibody, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit 1:500 in blocking solution) and ABC complex (Vectastain Elite ABC Kit, Vector Laboratories, Inc.) each for 30 min. Diaminobenzidine (DAB) was used as a chromogen to visualize the sites expressing activated caspase-3 immunoreactivity. The control sections were incubated without the primary antibodies to rule out nonspecific staining. Finally, the sections (without additional counter-staining) were dehydrated and mounted.

16.2.3 Semi-Quantitative Analysis of Caspase-3

The sections were processed under standardized conditions in every experiment, which allowed semi-quantitative analysis of the protein amount in the histological slices (Smolen 1990). An image analysis system comprising IBM PC, Nikon

Microphot-FXA microscope, SensiCam digital camera (PCO Computer Optics GmbH), Image-Pro Plus (Media Cybernetics) was used for a semi-quantitative analysis of caspase-3 expression in the histological sections of the cerebellum. Five sections cut at the same level of the cerebellum vermis from every animal were analyzed. The sections were reviewed at 250-fold magnification under a light microscope. The optical density was evaluated by two parameters reflecting the expression level of this protein in the lobules of the cerebellum. As the first parameter, the number of caspase-3-immunoreactive (IR) cells was calculated in every slice in each lobule and the average number of activated caspase-3-IR cells per slice counted. As the second parameter, the relative optical density of DAB precipitates in the perikaryons of individual cells was estimated in every section and the average optical density with its SEM calculated. Optical density was analyzed by the software as a "grey level" (GL). The optical density reflecting the content of the proteins studied in neurons was calculated as the GL of an IR field of the cell by subtracting the background GL. The optical density of the background was estimated in the same slice in the field of non-immunoreactive cerebellar tissue.

16.2.4 Detection of Cell Death In Situ

DNA fragmentation is one of the most precise markers by which to recognize apoptotic cells in tissue. In order to detect DNA fragmentation in 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) 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 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 DAB for 10 min at room temperature and then counterstained with hematoxylin-eosin.

16.2.5 Statistical Analysis

Statistical significance was determined by Student's *t* test. Each value was expressed as mean ± SEM. Differences were considered significant when the calculated p value was <0.05.

16.3 Results

16.3.1 Effects of Taurine on Caspase-3 Activation

There were randomly activated caspase-3-IR cells visible in the control group, reflecting the rate at which spontaneous (physiological) cell death occurs at this

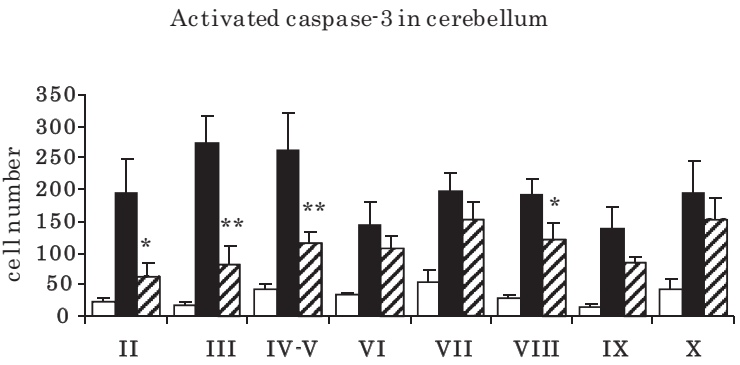

Fig. 16.1 The number of activated caspase-3-IR cells in the cerebellar lobules in control (*open bars*), ethanol-treated (*filled bars*) and ethanol+taurine-treated (*hatched bars*) mice. The results are given per mm^2 with SEMs. The number of animals in each group is 5. The significance of differences between ethanol and ethanol+taurine groups: $^{*}P < 0.05$, $^{**}P < 0.01$

age (Fig. 16.1). Following ethanol administration, there was a marked increase in activated caspase-3-IR granular neurons in all cerebellar lobules, indicating that ethanol triggered apoptotic neurodegeneration. Taurine treatment tended to reduce the number of activated caspase-3-IR cells in all cerebellar lobules when compared to the ethanol-treated group, the effect being significant in lobules II, III, IV-V and VIII, but it did not abolish it totally in any lobule. The content of activated caspase-3 in individual immunoreactive neurons was approximately the same in all experimental groups in all cerebellar lobules (Fig. 16.2).

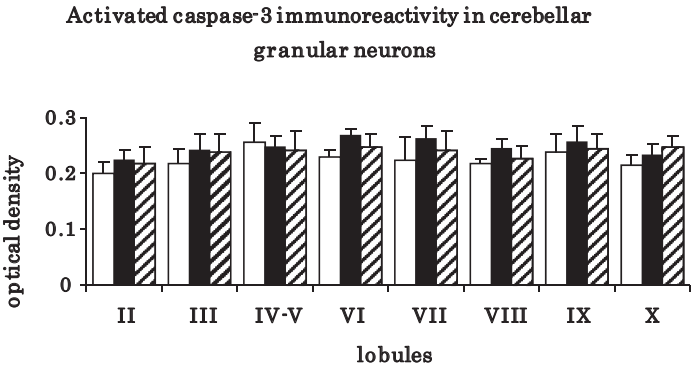

Fig. 16.2 The content of activated caspase-3 in individual cells in the cerebellar lobules in control (*open bars*), ethanol-treated (*filled bars*) and ethanol+taurine-treated (*hatched bars*) mice. The results show the optical density with SEMs. The number of animals in each group is 5

16.3.2 Effects of Taurine on Ethanol-Induced Apoptosis

TUNEL-positive cells were counted in each lobule of the cerebellar vermis (Fig. 16.3). Occasional TUNEL-positive cells were observed in the cerebellar lobules of control mice. Eight hours after ethanol administration the number of cells with fragmented DNA labeled by TUNEL assay was significantly increased in all lobules. In mice treated with taurine, the tendency towards a decrease in TUNEL-positive neurons was discernible in all cerebellar lobules except lobule VI, being statistically significant in lobules III, IV-V, VIII and IX.

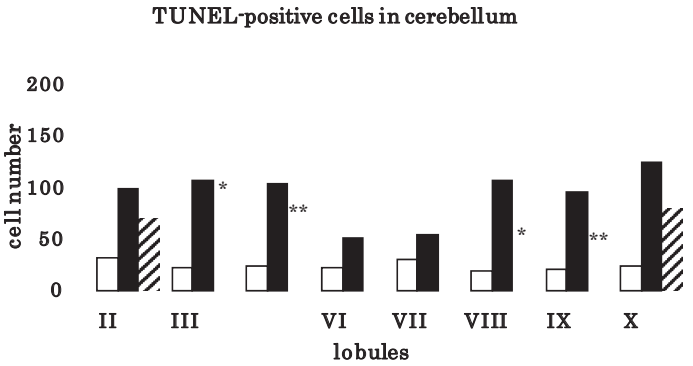

Fig. 16.3 The number of TUNEL-positive cells in the cerebellar lobules in control (*open bars*), ethanol-treated (*filled bars*) and ethanol+taurine-treated (*hatched bars*) mice. The results are given per mm^2 with SEMs. The number of animals in each group is 5. The significance of differences between ethanol and ethanol+taurine groups: $^{*}P < 0.05$, $^{**}P < 0.01$

16.4 Discussion

As already stated in the Introduction, acute exposure of rodents to ethanol during the period of developmental synaptogenesis causes extensive apoptotic neurodegeneration throughout the brain (Olney et al. 2000, 2002a). The degenerating neurons exhibit biochemical and ultrastructural features of apoptosis such as activation of caspase-3 (Olney et al. 2002a, 2002b) internucleosomal DNA fragmentation (Ikonomidou et al. 2000; Kumral et al. 2005) clumping of nuclear chromatin, formation of spherical chromatin masses and nuclear membrane fragmentation (Ikonomidou et al. 2000; Dikranian et al. 2001).

Two major apoptotic pathways have been established, one known as “extrinsic” and the other as “intrinsic” (Boatright and Salvesen 2003). In both pathways, the caspases play an important role in initiation, signal transduction and execution of apoptosis. The extrinsic pathway is triggered by activation of death receptors localized on the cell membrane surface and induces caspase-8 processing. The activated caspase-8 can directly or indirectly activate effector caspases such as

caspases-3, -6 and -7. In the intrinsic pathway, many factors such as NO, oxidants and proapoptotic proteins, e.g. Bax, increase the mitochondrial membrane permeability and release cytochrome C into the cytoplasm. Cytochrome C binds to Apaf-1 and procaspase-9, forming an apoptosome and leading to caspase-9 activation (Purring-Koch and McLendon 2000). The active caspase-9 cleaves and activates effector caspases, including caspase-3. The activated effector caspases cleave many structural and functional proteins, activate DNase, which destroys chromosomes and leads to cell death (Budihardjo et al. 1999; Earnshaw et al. 1999). Recent studies have shown that caspases-6, -7 and -8 are not involved in ethanol-induced apoptosis (Olney et al. 2002a; Young et al. 2003). Apoptosis caused 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).

Taurine is a sulfur-containing amino acid with multiple functions, including neuroprotection (Oja and Saransaari 2007). Earlier investigations on the interactions of taurine and ethanol in the brain have indicated that responses depend largely on the experimental set-up and the doses of ethanol and taurine administered (Oja and Saransaari 2007). The protective effects of taurine are not limited to ethanol. For instance, it has been shown in studies in vitro that taurine also protects cardiomyocytes (Takatani et al. 2004) and hypothalamic neurons (Taranukhin et al. 2007) from apoptosis induced by ischemia. Taurine suppresses the formation of the Apaf-1/caspase-9 apoptosome in cardiomyocytes and thereby prevents caspase-9 activation and apoptosis (Takatani et al. 2004). In our previous experiments we have shown that taurine reduces caspase-9 expression in hypothalamic neurons under ischemic conditions (Taranukhin et al. 2007). These findings and the knowledge that ethanol-induced apoptosis emerges preferentially via the mitochondrial pathway were the impetus to use taurine as a possible neuroprotector against ethanol-induced apoptosis. The present experimental set-up has been used as the model of ethanol-induced neurodegeneration (Ikonomidou et al. 2000; Olney et al. 2002a; 2002b; Young et al. 2003, 2005; Kumral et al. 2005; Nowoslawski et al. 2005), the time-points of which are well-defined. Because caspase-3 activation is considered an important step in the execution of apoptotic neuronal death and activated caspase-3 is widely expressed after acute ethanol administration (Olney et al. 2002a, b), the immunocytochemical detection of activated caspase-3 was adopted as a marker of apoptosis.

In the cerebellar lobules of control mice we saw only rarely activated caspase-3-IR neurons undergoing physiological cell death. As could be expected, eight hours after ethanol administration an increase in the number of activated caspase-3-IR cells was detected throughout all lobules. Taurine application reduced the number of caspase-3-IR neurons in the cerebellar lobules, but did not abolish them completely. The tendency towards a decrease in caspase-3-IR neurons was observed in all cerebellar lobules but taurine treatment did not alter the content of activated caspase-3 in individual immunoreactive neurons. The absence of graded effects suggests that taurine completely abolishes the activation of caspase-3 by ethanol in only one group of neurons, but has no effect in the others.

In the light of recent publications (Oomman et al. 2004; Rosado et al. 2006) caspase-3 activation is not always related to apoptosis. Furthermore, caspase-3

activation is a prominent feature but it is not an essential step in developmental ethanol-induced neuroapoptosis (Young et al. 2005). We therefore used an additional marker for apoptotic cells, DNA fragmentation using TUNEL staining. Only few cells with fragmented DNA labeled by TUNEL assay were found in the cerebellar lobules in control mice. Eight hours after ethanol administration an increase in the number of TUNEL-positive cells was observed throughout all vermis lobules. The picture of TUNEL staining in the cerebellum under ethanol effects was very similar to that of caspase-3-IR, confirming caspase-3 involvement in ethanol-induced developmental neuronal apoptosis. In the cerebella of ethanol-exposed taurine-treated mice, the number of apoptotic cells decreased significantly when compared to the ethanol-exposed mice. The number of caspase-3-positive neurons was greater than that of TUNEL-positive neurons, because caspase-3 activation preceeds eventual cell death.

We here show that taurine can rescue immature neurons from apoptosis induced by acute ethanol administration by suppressing activation of caspase-3. Which are the possible mechanisms of this? It might be supposed that taurine interferes with the Bax-dependent mitochondrial pathways of ethanol-induced apoptosis (Young et al. 2003), prevents apoptosome formation and caspase-9 activation (Takatani et al. 2004) and in this manner blocks caspase-3 activation and cell death. In this case however, the question remains why taurine blocks the apoptosis and caspase-3 activation only in some neurons, but does not completely abolish cell death. One possible answer is that ethanol induces apoptosis via several different mechanisms in different populations of neurons. Then, in those neurons in which ethanol induces apoptosis via caspase-9-dependent pathways taurine can have a neuroprotective effect and blocks caspase-9-dependent caspase-3 activation. On the other hand in other neurons in which caspase-3 activation is independent from caspase-9, taurine does not have an antiapoptotic effect. This assumption requires further experiments using specific blockers of the apoptotic proteins involving caspase-3 activation.

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