ANDREY G. TARANUKHIN

Janus-faced Taurine

Neuroprotection and Toxicity

ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the School of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
School of Medicine of the University of Tampere,
Medisiinarinkatu 3, Tampere, on November 8th, 2013, at 12 o’clock.
To my family

"All things are poison, and nothing is without poison; only the dose permits something not to be poisonous."

Paracelsus 1493–1541 (Philippus Aureolus Theophrastus Bombastus von Hohenheim)
1. Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>2. Abstract</td>
<td>7</td>
</tr>
<tr>
<td>3. Abbreviations</td>
<td>9</td>
</tr>
<tr>
<td>4. List of Original Communications</td>
<td>11</td>
</tr>
<tr>
<td>5. Introduction</td>
<td>12</td>
</tr>
<tr>
<td>6. Review of the Literature</td>
<td>13</td>
</tr>
<tr>
<td>6.1 Taurine</td>
<td>13</td>
</tr>
<tr>
<td>6.1.1 History of Taurine</td>
<td>13</td>
</tr>
<tr>
<td>6.1.2 Taurine Distribution and Metabolism</td>
<td>13</td>
</tr>
<tr>
<td>6.1.3 Physiological Actions of Taurine</td>
<td>18</td>
</tr>
<tr>
<td>6.1.3.1 Osmoregulation</td>
<td>18</td>
</tr>
<tr>
<td>6.1.3.2 Conjugation with Bile Acids</td>
<td>18</td>
</tr>
<tr>
<td>6.1.3.3 Detoxification</td>
<td>19</td>
</tr>
<tr>
<td>6.1.3.4 Neuromodulation and Neurotransmission</td>
<td>19</td>
</tr>
<tr>
<td>6.1.3.5 Maintenance of Calcium Homeostasis</td>
<td>21</td>
</tr>
<tr>
<td>6.1.4 Role of Taurine in Development</td>
<td>22</td>
</tr>
<tr>
<td>6.1.5 Taurine in Cell Protection and Apoptosis Regulation</td>
<td>23</td>
</tr>
<tr>
<td>6.1.6 Taurine in Health and Disease</td>
<td>24</td>
</tr>
<tr>
<td>6.2 Cell Death</td>
<td>27</td>
</tr>
<tr>
<td>6.2.1 Cell Death Modalities</td>
<td>27</td>
</tr>
<tr>
<td>6.2.2 Apoptosis</td>
<td>30</td>
</tr>
<tr>
<td>6.2.3 Caspases in Apoptosis</td>
<td>31</td>
</tr>
<tr>
<td>6.2.4 Pathways of Apoptosis</td>
<td>32</td>
</tr>
<tr>
<td>6.2.4.1 Extrinsic or Receptor-Mediated Apoptosis</td>
<td>32</td>
</tr>
<tr>
<td>6.2.4.2 Intrinsic or Mitochondria-Mediated Apoptosis</td>
<td>33</td>
</tr>
<tr>
<td>6.2.5 Significance of Apoptosis in Health and Disease</td>
<td>34</td>
</tr>
<tr>
<td>6.2.6 Apoptosis in Ischemia</td>
<td>37</td>
</tr>
<tr>
<td>6.2.7 Apoptosis in Fetal Alcohol Syndrome</td>
<td>38</td>
</tr>
<tr>
<td>7. Aims of the Study</td>
<td>41</td>
</tr>
</tbody>
</table>
8. Materials and Methods ........................................................................................................ 42
  8.1 Animals (I-V) .................................................................................................................. 42
  8.2 Ethical Considerations ................................................................................................... 42
  8.3 Experimental Models .................................................................................................... 42
    8.3.1 Ischemia-Induced Apoptosis In Vitro (I) .......................................................... 42
    8.3.2 Ethanol-Induced Apoptosis In Vivo (II-IV) ..................................................... 43
    8.3.3 Combined Toxicity of Ethanol and Taurine In Vivo (V) ................................. 44
  8.4 Conventional Histology (I-IV) .................................................................................... 45
  8.5 Immunohistochemistry (I-IV) .................................................................................. 45
  8.6 Detection of Cell Death In Situ (II-IV) ...................................................................... 46
  8.7 Microscopy, Image Analysis and Cell Counting .................................................... 46
  8.8 Semi-Quantitative Analysis of Caspases ................................................................ 47
  8.9 High-Performance Liquid Chromatography (HPLC) (III) ................................. 48
  8.10 Blood Glucose Determination (V) ......................................................................... 48
  8.11 Data Expression and Statistical Analysis .................................................................. 49
9. Results .................................................................................................................................. 50
  9.1 Effect of Taurine on Ischemia-Induced Expression of Caspase-8 and Caspase-9 (I) .................................................................................................................. 50
  9.2 Effect of Taurine on Ethanol-Induced Expression of Activated Caspase-3 (II, III, IV) .................................................................................................................. 50
  9.3 Effects of Taurine on Ethanol-Induced Apoptosis .................................................. 52
  9.4 Dynamics of Taurine Blood Concentration Changes During the Experiments (III) .................................................................................................................. 53
  9.5 Combined Toxicity of Taurine and Ethanol in 7-Day-Old Mice (V) .......................... 53
  9.6 Combined Toxicity of Taurine and Ethanol in Adult Mice (V) ............................... 54
  9.7 Combined Toxicity of Taurine and Ethanol in Old Mice (V) ....................................... 54
  9.8 Changes in Blood Glucose Level after Taurine and Ethanol Co-Administration in Adult Mice (V) ........................................................................................................ 55
  9.9 Changes in Blood Glucose Level after Taurine and Ethanol Co-Administration in Old Mice (V) ........................................................................................................ 55
10. Discussion .......................................................................................................................... 56
  10.1 Methodological Aspects ............................................................................................ 57
  10.2 Intracellular Mechanisms of the Anti-Apoptotic Effects of Taurine ....................... 59
  10.3 Combined Toxicity of Taurine and Ethanol and Its Possible Mechanisms ............ 64
11. Summary ............................................................................................................................ 69
12. Conclusions and Implications

13. Acknowledgements

14 References

15 Original Communications
2. Abstract

Ischemia and heavy alcohol consumption can cause brain damage due to the loss of substantial numbers of neurons, this leading to neurodegenerative diseases and disability. Apoptosis, one of the major types of programmed cell death, contributes significantly to this neuronal destruction. This mode of cell death is however a tightly regulated process in which the consecutive events go step by step, involving activation of many specific proteins which gradually destroy the cell, and the stagewise nature of this degradation gives a possibility by clinical intervention to save neurons from apoptosis and to reduce significantly the serious consequences of ischemia and excessive alcohol consumption. Among candidate drugs in this context the amino acid taurine seems particularly attractive, since it is a naturally occurring non-toxic compound abounding in the nervous system. It is involved in a wide range of physiological processes such as osmoregulation, lipid metabolism, intracellular calcium regulation, neuronal development, neuromodulation and cell protection. This study describes the protective effects of taurine against apoptotic cell death in ischemia- and alcohol-induced brain damage as well as the toxic effect of a combination of large doses of taurine with alcohol.

In the in vitro experiments conducted here on adult mice taurine (20 mM) reduced the expression of apoptotic signal proteins caspase-8 and caspase-9 in hypothalamic neurons under ischemic conditions. Then, using specific markers of apoptotic cell death, caspase-3 and TUNEL in 7-day-old mice, taurine (2 g/kg) protected immature granular neurons against ethanol-induced apoptosis. In experiments on 4-day-old mice Purkinje cells were not saved by taurine. On the other hand, neurons in the internal granular layer of the cerebellum were protected. These neuroprotective effects thus clearly depend on the type of neuron involved and may be influenced by the rate of taurine excretion into the urine. The neuroprotective effect of taurine in alcohol-induced apoptosis was also confirmed with neurons from the external layer of the cerebellum.
On the other hand, any further increase in taurine doses (4-6 g/kg) aiming to protect more neurons against alcohol-induced apoptosis poses to threat to the whole organism and kills 7-day-old mice thus treated. Such doses of taurine and ethanol led to the death of 50 % and 100 % of 7-day-old mice. The experiments on adult and old mice showed that one reason for this toxicity may be hypoglycemia.

Based on the results obtained we conclude that taurine may have beneficial effects in protecting brain cells against the apoptosis induced by ischemia and alcohol. Taurine is involved in apoptosis regulation at different stages. It reduces the expression of initiator caspases-8 and -9 and blocks the activation of executive caspase-3. As a result, DNA fragmentation and cell death are prevented. These observations constitute an incentive to search for the optimal strategy for taurine treatment. However, our finding on the toxicity of combined taurine and ethanol prompts serious concern particularly for young people mixing taurine-containing energy drinks with alcohol.
3. Abbreviations

ACSF  artificial cerebral spinal fluid
ADNF-9  peptide derived from activity-dependent neurotrophic factor
ANOVA  analysis of variance
Apaf-1  apoptotic protease activating factor-1
CNS  central nervous system
DAB  diaminobenzidine
DD  death domain
DED  death effector domain
EGL  external granular layer
ER  endoplasmic reticulum
FADD  Fas-associated death domain protein,
FAS  fetal alcohol syndrome
GL  grey level of optical density
HPLC  high-performance liquid chromatography
IGL  internal granular layer
IR  immunoreactive
NMDA  N-methyl-D-aspartate
NMRI-mice  Naval Medical Research Institute mice
OPA  o-phthaldialdehyde
P0  day of birth
P4  postnatal day 4
P6  postnatal day 6
P7  postnatal day 7
P8  postnatal day 8
P21  postnatal day 21
PBS  phosphate-buffered saline
PVN  paraventricular nucleus
ROS  reactive oxygen species
SON      supraoptic nucleus
SOD      superoxide dismutase
TNF-R-1  tumor-necrosis factor receptor
TUNEL    terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
wt       weight
4. List of Original Communications

This thesis is based on the following communications, which are referred to in the text by their Roman numerals I-V.


The original publications are reproduced with permission of the copyright holders.
5. Introduction

Taurine is a unique molecule with a variety of functions in the body. This small simple molecule participates, for example, in osmotic regulation, bile acid conjugation, detoxification, membrane stabilization, regulation of intracellular calcium homeostasis, neuromodulation and cell protection.

Taurine is almost absent in all plants but exists at high concentrations throughout the animal kingdom. Its highest concentrations are encountered in electrically excitable tissues such as muscles, brain and heart. It is not generally considered an essential amino acid and it is not incorporated in proteins. However, taurine is vitally important to most animals; for example, without taurine cats are blinded and polar bears suffer from metabolic bone disease and rickets. On the other hand, other animal species such as the guinea pig exhibit complete viability with a minimal concentration of taurine in the body.

Taurine has been tested for the therapy of a wide range of diseases, e.g. epilepsy, cardiovascular disorders, diabetes and alcoholism. It is generally considered safe and nontoxic. However, it may be harmful if consumed in conjunction with alcohol. Until recently taurine was interesting only to a small group of scientists around the world. Nowadays, however, thanks to commercial interest and intensive marketing, it is a common constituent in shampoos, creams, various dietary supplements and energy drinks. These latter are particularly popular among schoolchildren.

The present study was undertaken to bring out the beneficial effects of taurine in neuroprotection. It was hoped to lay the way for a search for an optimal treatment strategy in many neurological disorders. However, in the course of studies on the protective functions of taurine, unexpected evidence emerged toxicity when combined with alcohol. The focus thus shifted to these two facets of taurine.
6. Review of the Literature

6.1 Taurine

6.1.1 History of Taurine

Taurine (2-aminoethanesulphonic acid) is a simple sulphur-containing amino acid ubiquitously distributed in the tissues of most animals, including mammals (Jacobsen and Smith, 1968). A human weighing 70 kg contains up to 70 g of taurine (Huxtable, 1992). Taurine is not incorporated into tissue proteins and is thus present as a free amino acid. It occurs at higher concentrations than other amino acids in tissues such as muscle, platelets, retina and the central nervous system. Taurine was discovered in 1827 by the German scientists Friedrich Tiedemann and Leopold Gmelin, who first isolated it from ox bile (Tiedemann and Gmelin, 1827; Jacobsen and Smith, 1968). The compound was named by Demarcay in 1838 after the Latin word taurus, which means bull or ox (Demarcay, 1838; Lombardini and Schaffer, 1998). The first extensive review article on taurine was published by Jacobsen and Smith in 1968, whereafter scientific interest for this amino acid began to grow and is still growing. This is not surprising in that this molecule has a wide range of physiological functions and is involved in many processes in living organisms, including neurodegeneration (Dawson, 2003), aging (El Idrissi et al., 2009), cytoprotection (Timbrell et al., 1995), apoptosis (Condron et al., 2003), cancer (El Agouza et al., 2011), diabetes (Kim et al., 2007), epilepsy (Airaksinen et al., 1980), neurotransmission (Saransaari and Oja, 2008), and many others.

6.1.2 Taurine Distribution and Metabolism

Taurine is present in bacteria (Cook and Denger, 2006) and sporadically in plants at low concentrations (μmol/kg wet weight (wt)) (Huxtable, 1992). It is found throughout the animal kingdom at fairly high concentrations (mmolk/g wet wt)
(Jacobsen and Smith, 1968). The taurine contents in tissues vary markedly between different animal species. For example, its concentration in the brain of guinea pigs is rather low (less than 1 mmol/kg) and high in the rat brain (about 5 mmol/kg) (Oja et al., 1968). The content also varies among different tissues within one animal species (Jacobsen and Smith, 1968), being, for instance, low in the rat adrenals (1.9 mmol/kg wet wt) and high in the rat bone marrow (13.7 mmol/kg wet wt). Again, for example, the taurine content during development is 13.5 µmol/g and 5.2 µmol/g in the brain of 7-day-old and adult mice, respectively (Oja et al., 1976). Its distribution in the human body is shown in Table 1 (Lourenço and Camilo, 2002).

**Table 1. Taurine distribution in the human body**

<table>
<thead>
<tr>
<th></th>
<th>mmol/kg (wet weight)</th>
<th>µmol/l (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina..............</td>
<td>30-40</td>
<td>Milk.......... 337</td>
</tr>
<tr>
<td>Cells..............</td>
<td>20-35</td>
<td>Bile........... 200</td>
</tr>
<tr>
<td>White blood...</td>
<td>20-35</td>
<td>Saliva....... 16-65</td>
</tr>
<tr>
<td>Platelets..........</td>
<td>16-24</td>
<td>Liquor...... 5-36</td>
</tr>
<tr>
<td>Spleen.............</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Heart..............</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Muscle............</td>
<td>2.2-5.4</td>
<td></td>
</tr>
<tr>
<td>Brain...............</td>
<td>0.8-5.3</td>
<td></td>
</tr>
<tr>
<td>Lung...............</td>
<td>1-5</td>
<td></td>
</tr>
<tr>
<td>Kidney.............</td>
<td>1.4-1.8</td>
<td></td>
</tr>
<tr>
<td>Liver..............</td>
<td>0.3-1.8</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes......</td>
<td>0.05-0.07</td>
<td></td>
</tr>
<tr>
<td>Milk..............</td>
<td>337</td>
<td></td>
</tr>
<tr>
<td>Bile..............</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Saliva..............</td>
<td>16-65</td>
<td></td>
</tr>
<tr>
<td>Liquor.............</td>
<td>5-36</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes......</td>
<td>0.05-0.07</td>
<td></td>
</tr>
</tbody>
</table>

Taurine is one of the most abundant free amino acids in the brain, its concentration being exceeded only by that of glutamate (Oja and Saransaari, 2007). In the retina its concentrations are even higher (Huxtable, 1989). It seems that all cell types in the brain contain taurine; it is ubiquitously present for example in the rat brain, though its distribution is uneven in different brain areas. High taurine levels have been found in cerebral cortical areas, caudate-putamen, cerebellum, median eminence and the supraoptic nucleus of the hypothalamus (Palkovits et al., 14)
Taurine-like immunoreactivity has been demonstrated by taurine-specific antibodies in many structures and cell types in the brain (Yoshida et al., 1986; Ottersen, 1988; Ottersen et al., 1988). For example, taurine-like immunoreactivity was found in neurons of the rat dentate gyrus and the CA1, CA3 and CA4 regions of the hippocampus (Magnusson et al., 1989). In the rat cerebellum this immunoreactivity was concentrated in Purkinje cells (Yoshida et al., 1986; Ottersen et al., 1988; Magnusson et al., 1988; Pirvola and Panula, 1992; Zhang and Ottersen, 1992) while only weak immunoreactivity was present in Golgi, stellate and basket cells (Magnusson et al., 1988; Ottersen et al., 1988). The concentration of taurine thus varies considerably among the different cell types, but may also differ between different parts of the same neuron; for example, the Purkinje cell terminals evince 60% higher taurine-like immunoreactivity than Purkinje somata (Ottersen, 1988). Glial elements of the cerebellum demonstrate low taurine-like immunoreactivity (Zhang and Ottersen, 1992), whereas in the supraoptic nucleus of the hypothalamus taurine-like immunoreactivity is localized in glial cells (Decavel and Hatton, 1995). Interesting data have been obtained from cats. In neonatal cats Purkinje cells demonstrate strong taurine-like immunoreactivity, while in the mature cerebellum Purkinje cells contain only GABA and glutamate and virtually no taurine, in contrast to findings on the rodent cerebellum (Lu et al., 1993).

The extracellular concentrations of taurine have been measured in only a few structures in the rat brain and the results are comparable, 20.6 µM in the dentate gyrus (Lerma et al., 1986) and 25.2 µM in the striatum (Molchanova et al., 2004), for instance. The intracellular concentration of taurine exceeds its extracellular concentration about 400 times. In this respect taurine occupies an intermediate position between neuroactive (intra/extracellular concentration ratio more than 2000) and nonneuroactive amino acids (intra/extracellular concentration ratio less than 100) (Lerma et al., 1986).

There are two sources from which animals acquire taurine. It can be obtained from consumed food of animal origin, for example meat and seafood, and it can also be synthesized from two sulphur-containing amino acids, methionine and cysteine, which serve as taurine precursors. Two major pathways of taurine synthesis are possible (Fig. 1). In the first, cysteine is oxidized to cysteine sulfinate by cysteine dioxygenase, which is then decarboxylated by sulfinoalanine decarboxylase to hypotaurine (Oja and Kontro, 1983a; Huxtable, 1992). In the second more
complicated pathway cysteine is converted via several steps to coenzyme A and then to pantetheine. Pantetheine is then transformed to cysteamine and finally to hypotaurine by cysteamine dioxygenase (Coloso et al., 2006). As the final step in both of these major pathways of taurine synthesis, hypotaurine is possibly oxidized to taurine by nonenzymatic oxidation (Fellman and Roth, 1985), although the involvement of enzymes has also been considered (Oja and Kontro, 1981; Kontro and Oja, 1985). While a putative hypotaurine dehydrogenase has not been purified to homogeneity, some recent findings strongly suggest that the conversion of hypotaurine to taurine is enzymatically catalyzed, and that this oxidation of hypotaurine limits taurine synthesis in cells (Vitvitsky et al., 2011).

Although the brain contains all enzymes necessary for taurine biosynthesis, the process seems to be very slow (Beetsch and Olson, 1998) in comparison with the active uptake of taurine by brain cells via plasma membrane transport (Lähdesmäki and Oja, 1973; Beetsch and Olson, 1996). The rat brain enzymes for taurine biosynthesis do not respond to a dietary supply of the precursors methionine and cysteine (Stipanuk et al., 2002), whereas their activity in the rat liver and hepatocytes is strongly upregulated by them (Stipanuk et al., 2002; Tappaz, 2004). It would thus appear that the taurine levels in the brain depend mainly on the blood supply from other tissues, particularly from the liver (Oja and Saransaari, 2007). The capacity for taurine biosynthesis varies between species (Worden and Stipanuk, 1985). It is believed to depend on the activity of sulfinoalanine decarboxylase, the rate-limiting enzyme responsible for the formation of taurine from cysteine. The activity of this enzyme is significantly higher in the rat when compared for instance to the cat (de la Rosa and Stipanuk, 1985). Bacteria are able to synthesize taurine from simple building elements and to break it down (Cook and Denger, 2006). Mammalian tissues, however, are not able to reduce sulphur-containing compounds. They can only excrete taurine or first conjugate and then excrete it. The kidneys excrete taurine into the urine, the rate of excretion depending on the dietary intake and the plasma levels of taurine (Chesney et al., 1985).
Fig.1. Metabolism of taurine in mammalian tissues. Only the most important intermediate products are shown. Adapted from Huxtable, 1992; Lourenço and Camilo, 2002; Coloso et al., 2006; Vitvitsky et al., 2011.
6.1.3 Physiological Actions of Taurine

6.1.3.1 Osmoregulation

The maintenance of appropriate volumes is one important condition for cell survival (Lang et al., 1998a). Excessive changes in cell volume interfere with the integrity of cell membranes and cytoskeletal architecture. The most rapid and efficient cell volume-regulatory mechanisms are ion transporters in the cell membrane (Lang et al., 1998b). Following cell swelling, they mediate cellular ion release and upon cell shrinkage allow cellular ion accumulation. The efficacy of ions in cell volume regulation is limited, however, as high inorganic ion concentrations interfere with the stability of proteins and the altered ion gradients across the cell membrane interfere with the function of gradient-driven transporters (Garcia-Perez and Burg, 1991). The cells therefore additionally use organic osmolytes for osmoregulation, and one of these is taurine. In the central nervous system (CNS) taurine may play a dominant role in the regulation of cell volumes. A release of taurine from cultured glial cells and neurons under hypoosmotic conditions has been demonstrated (Pasantes-Morales and Schousboe, 1988; Pasantes-Morales et al., 1990; Oja and Saransaari, 1992a, b). Cell-volume regulation is the phylogenetically oldest function of taurine, conserved from amoebas to mammals (Huxtable, 1992).

6.1.3.2 Conjugation with Bile Acids

Bile acids function as detergents for the emulsification of lipids and fat-soluble vitamins and aid their absorption. Bile salts are critical for this function in that the lipophilic and hydrophilic components in their molecules can lower the surface tension of lipids to form micelles. The liver synthesizes primary bile salts cholate and chenodeoxycholate from cholesterol. The intestinal bacteria convert some of them into secondary bile salts such as deoxycholate and lithocholate. The bile salts are conjugated with taurine or glycine in the liver and secreted into the bile in these conjugated forms. This conjugation is essential for micelle formation (Silbernagl and Despopoulos, 2009). For example, taurine is a key for the conjugation of ursodeoxycholic acid, a bile acid unique to bears. Taurine-conjugated
ursodeoxycholic acid optimizes fat and fat-soluble vitamin absorption. Taurine deficiency causes vitamin D malabsorption and leads to metabolic bone disease and rickets in polar bear cubs raised in captivity (Chesney et al., 2009). Taurine conjugation of bile acids has a significant effect on the solubility of cholesterol, increasing its excretion. Daily administration of 6 g of taurine has been shown to reduce serum cholesterol levels in human subjects (Mizushima et al., 1996). Furthermore, it is assumed that a high taurine level in the blood may be protective against coronary heart disease among subjects with high serum cholesterol levels (Wójcik et al., 2013).

6.1.3.3 Detoxification

Detoxification is a process which modifies endogenous waste products and foreign compounds (xenobiotics) into a less toxic form and prepares them for rapid elimination. Animal studies have demonstrated ability of taurine to complex with and neutralize the xenobiotic effects of carbon tetrachloride (Nakashima et al., 1982; Waterfield et al., 1993; Wu et al., 1997). Taurine has been found to inhibit significantly intestinal endotoxin translocation, subsequently reducing hepatic injury and protecting from endotoxemic injury (Wang, 1995; Roth et al., 1997). It has been shown that taurine reacts with and detoxifies the hypochlorous acid generated by neutrophils from myeloperoxidase, hydrogen peroxide and chloride during an oxidative burst. This is a protective function which involves the formation of stable taurochloramine, and it is believed to be the reason for the high levels of taurine (50 mM) in neutrophils (Timbrell et al., 1995). It is further assumed that taurine has a potential in the treatment of alcoholic liver fibrosis, this in view of the positive effects of taurine treatment on the metabolism and detoxification of ethanol in rat liver fibrosis induced by simultaneous administration of iron carbonyl and ethanol (Devi et al., 2009).

6.1.3.4 Neuromodulation and Neurotransmission

It is thought that taurine can act in the CNS as a neurotransmitter or a neuromodulator (Oja et al., 1977; Oja and Kontro, 1983; Oja and Saransaari, 1996;
Frosini et al., 2003; El Idrissi and Trenkner, 2004; Saransaari and Oja, 2008). Taurine does not activate ionotropic glutamate receptors [2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoate (AMPA) and N-methyl-D-aspartate (NMDA) receptors] (Song et al., 2012). Owing to its inhibitory propensities it may counteract any excitotoxicity caused by the excessive glutamate release in ischemia, hypoglycemia and other cell-damaging conditions (Saransaari and Oja, 1991, 1998). Inhibitory responses to taurine include membrane hyperpolarization, reduction in membrane input resistance and suppression of spontaneous firing (Belluzzi et al., 2004; Chaput et al., 2004). It has been shown that taurine interacts with $\text{GABA}_A$ (Malminen and Kontro, 1987; Quinn and Harris, 1995) and $\text{GABA}_B$ (Kontro and Oja, 1990; Kontro et al., 1990) receptors, acting as their agonist. Taurine also interacts with glycine receptors (Kontro and Oja, 1987; Xu et al., 2004). For example, in the rat supraoptic magnocellular neurons taurine acts as the agonist on the glycine receptors (Hussy et al., 1997). Sometimes, taurine exerts its effects through both GABA and glycine receptors simultaneously. It controls hormone release from the rat neurohypophysis by acting via both $\text{GABA}_A$ receptors and one or more glycine receptors (Song and Hatton, 2003). In some brain areas activation by taurine of $\text{GABA}_A$ and/or glycine receptors depends on the taurine concentration. Thus, a moderate taurine concentration (0.3 mM) leads to activation of glycine receptors, whereas at a high taurine concentration (3 mM) it activates both glycine and $\text{GABA}_A$ receptors in rat substantia gelatinosa neurons (Wu et al., 2008). However, for instance in the rat anteroventral cochlear nucleus neurons taurine at low (0.1 mM) and high (1 mM) concentrations activates both $\text{GABA}_A$ and glycine receptors (Song et al., 2012). Moreover, in experiments where taurine and GABA or taurine and glycine were applied simultaneously, the response was greater than that evoked by GABA or glycine alone. These findings suggest that taurine can serve as a neuromodulator by way of increasing GABAergic and glycinerergic inhibitory neurotransmission (Song et al., 2012). The possible existence of specific taurine receptors has also been discussed in the case of certain brain regions, at developmental stages and in different animal species (Kudo et al., 1988; Wu et al., 1992; Frosini et al., 2003). However, no agonists or antagonists sufficiently specific for definite identification of possible taurine receptors have so far been found (Oja and Saransaari, 2007).
One interesting new finding is that taurine can exert its neuromodulatory effects when acting on the genetic level. Taurine given to mice in drinking water during 4 weeks has reduced the expression of β1, β2 and β3 subunits of the GABA\textsubscript{A} receptor in several brain regions. It is suggested that taurine-fed mice have elevated extracellular taurine levels, which lead to sustained activation or at least binding to GABA\textsubscript{A} receptors. Such chronic interaction of taurine with GABA\textsubscript{A} receptors may lead to down-regulation of GABA\textsubscript{A} receptor function or expression (Shen et al., 2013).

6.1.3.5 Maintenance of Calcium Homeostasis

The cytoplasmic calcium concentration modulates various cellular functions, for example gene expression, metabolism, proliferation, secretion, neural excitation and fertilization (Berridge et al., 2000; Tandogan and Ulusu, 2005). A delicate regulation of calcium homeostasis is required for cell survival and its perturbation results in cell death through both necrosis and apoptosis (Berridge et al., 2000; Orrenius et al., 2003). For example, in neurons calcium plays a key role in mediating glutamate excitotoxicity. Glutamate-induced neuronal necrosis is preceded by a rapid increase in the cytoplasmic free calcium concentration (Choi and Rothman, 1990).

It has been shown that taurine participates in calcium regulation. For example, taurine inhibits the glutamate-induced release of calcium from the internal pools (Wu et al., 2000) and regulates cytoplasmic and mitochondrial calcium homeostasis (Foos and Wu, 2002; El Idrissi and Trenkner, 2003). Taurine also inhibits the glutamate-induced calcium influx through the L-, N- and P/Q-types of voltage-gated calcium channels and the NMDA receptor calcium channel (Wu et al., 2009). In addition to blocking this calcium influx taurine is also able to reduce intracellular calcium in neurons by increasing the presence of calcium-binding proteins such as calbindin D28k and calretinin (Junyent et al., 2010). It is assumed that this ability of taurine to lower the intracellular calcium concentration is closely connected to its neuroprotective function (Wu et al., 2009; Junyent et al., 2010; Gao et al., 2011).
6.1.4 Role of Taurine in Development

It is well established that taurine is crucially required for normal development in mammals, especially for the CNS (Sturman, 1993; Aerts and Van Assche, 2002; Pasantes-Morales and Hernandez-Benitez, 2010). Taurine concentrations have been assessed in the brains of adult and newborn animals (mouse, rat, gerbil, guinea pig, rabbit, dog, cat, chick, monkey and human) and the results clearly demonstrate that the brain taurine concentration in the newborns of all animals studied is 1.7 - 5.2-fold greater than in the adults (Sturman, 1993). In the developing brain taurine may be even more important than in the adult brain, as it appears to be the only inhibitory effector when the GABAergic transmitter systems have not yet functionally matured (Saransaari and Oja, 1997; Oja and Saransaari, 2013a). Taurine deficiency impairs neuronal migration. A decrease in the intracellular taurine concentration by 60 % in mouse cerebellar cultures in vitro has reduced cell migration by 50% when compared to the normal taurine concentration (Maar et al., 1995). Cats have low levels of activity of sulfinoalanine decarboxylase (the rate-limiting enzyme for taurine biosynthesis) and conjugate only taurine, not glycine, with bile acids and are therefore dependent on a dietary source of taurine (Hardison et al., 1977; de la Knopf et al., 1978; Rosa and Stipanuk, 1985; Sturman et al., 1986).

Kittens nursed with low-taurine milk exhibit a lower growth rate than normal, have lower tissue taurine concentrations, lower brain weight and 8 weeks after birth evince a persistence of cells in the cerebellar external granule cell layer, which normally disappears within 3-4 weeks after birth (Smith and Downs, 1978; Sturman et al., 1985a, b). Daily oral supplementation with 40 µmoles taurine restores the growth rate almost to the level of normally nurtured kittens and results in normal tissue taurine concentrations and apparently normal migration of cells in the cerebellum (Sturman et al., 1985b). Another example shows that antenatal taurine treatment given at a dose of 300 mg/kg daily from 12 days onwards after conception until natural delivery significantly improves brain development in the rat fetus with intrauterine growth restriction. Taurine treatment reduces fetal mortality, and increases birth weight and brain weight (Liu et al., 2011). Taurine also protects cells from apoptosis (Liu et al., 2011, 2012) and promotes maturation of surviving neurons (Liu et al., 2011). Furthermore, taurine treatment increases the expression
of neurotrophic factors and promotes cell proliferation to counteract the neuron loss caused by intrauterine growth restriction (Liu et al., 2013). In experiments with mice and rats in vivo, ex-vivo and in vitro, it has been shown that taurine enhances proliferation of neural stem cells and increases neurite growth and synaptogenesis (Shivaraj et al., 2012). These findings could potentially indicate that the smaller brain weight and abnormal brain morphology seen in cats and monkeys born of taurine-deficient mothers (Sturman et al., 1985a; Sturman, 1993) could be due to a decreased proliferation of neural progenitor cells in the absence of taurine. In vitro in human fetal neurons taurine has been found necessary for neuronal survival and neurite extension. Taurine also exerts a trophic effect on human fetal brain cells, promoting both their proliferation and differentiation (Chen et al., 1998).

All the above findings have clear implications for vegetarian women who intend to have children, since virtually no taurine is present in plants and vegetables. On the other hand, no notable difference has been seen in the taurine concentrations in breast milk in vegans compared with omnivores. This would suggest a considerable capacity of adult humans to synthesize taurine and suppress urinary taurine excretion, during both pregnancy and lactation. It may be assumed that the physiological response to reproduction satisfies the needs of this amino acid in the fetus and the suckling infant (Rana and Sanders, 1986; Naismith et al., 1987).

6.1.5 Taurine in Cell Protection and Apoptosis Regulation

Many researchers have demonstrated that taurine protects several types of cells under a variety of pathological conditions. Taurine protects cardiomyocytes from ischemia- (Takahashi et al., 2003) and reperfusion-induced (Milei et al., 1992) damage as well as from diabetic cardiomyopathy (Li et al., 2005). It prevents the endothelial cell death induced by sodium arsenite and tumor necrosis factor-alpha (Wang et al., 1996), hyperglycemia (Ulrich-Merzenich et al., 2007) and methamphetamine-induced injury (Shao et al., 2012). Taurine also protects hepatocytes from acetaminophen-induced cell death (Waters et al., 2001). It protects the testes (Das et al., 2009) and kidney (Roy et al., 2009) against arsenic-induced damage. Furthermore, taurine rescues neurons from glutamate- (Leon et al., 2009) and morphine- (Zhou et al., 2011) induced cell death. It exerts a protective effect in
endotoxin-induced acute lung inflammation (Bhavsar et al., 2010) and protects the liver, somatic and germ cells and sperm from genotoxic damage induced by methotrexate and tamoxifen (Alam et al., 2011). Recent publications also show that taurine plays an important role in apoptosis regulation (Takatani et al., 2004; Sun and Xu, 2008; Wu et al., 2009). The mechanisms of the protective and antiapoptotic effects of taurine are considered in detail in the Discussion section.

6.1.6 Taurine in Health and Disease

Taurine is involved in a wide range of physiological processes. It also exerts cytoprotective and antiapoptotic actions against various harmful conditions. Taurine is thus an attractive compound for application in medicine (Kendler, 1989; Lourenço and Camilo, 2002). Indeed, taurine has been used in clinical practice for the treatment of a number of diseases, albeit with varying degrees of success (Birdsall, 1998; Della Corte et al., 2002). Several studies have shown that taurine is a safe and sometimes effective therapeutic tool in the management of various types of cardiovascular diseases (Xu et al., 2008; Wójcik et al., 2010). Taurine comprises over 50 % of the total free amino acid pool in the heart (Jacobsen and Smith, 1968). Oral administration of taurine leads to a decrease in blood pressure in animals (Nara et al., 1978; Abe et al., 1987) and humans (Fujita et al., 1987; Militante and Lombardini, 2002) as well as to a decrease in serum cholesterol levels in humans (Mizushima et al., 1996). In one double-blind, placebo-controlled cross-over study taurine proved effective in the treatment of patients with congestive heart failure, and the treatment had no side-effect (Azuma et al., 1985). It would appear that taurine exerts its cardioprotective effects (at least partly) due its ability to regulate intracellular and extracellular calcium, and is effective both at low calcium levels and during calcium overload (Satoh, 1994; Satoh and Sperelakis, 1998).

Taurine has been used in the treatment of patients with epilepsy. However, results have been conflicting, possibly due to methodological flaws (Fariello et al., 1985). In experimental conditions a taurine injection to mice at a dose of 43 mg/kg significantly reduces the occurrence of tonic seizures, the duration of tonic-clonic convulsions and the mortality rate following kainic acid-induced seizures (El Idrissi et al., 2003). A positive effect of taurine treatment in humans has been reported in
one cross-over double-blind placebo-controlled study (Airaksinen et al., 1980a). In this study taurine at a dose of 100 mg/kg was given orally for two months to patients with epilepsy. This treatment reduced the number of seizures in one third of drug-resistant patients by at least 50% without any side-effects. On the other hand, oral taurine treatment at doses from 16 to 150 mg/kg to patients with epilepsy during four to six months had no beneficial effects on the seizure frequency (Mantovani and DeVivo, 1979). In another study, nine patients with epilepsy received orally 1.5 – 7.5 g taurine daily over a period of 4 to 16 weeks, and despite this high dosage, no appreciable side-effects were observed. During this time seizures disappeared temporarily for about two weeks in 5 out of 9 cases. In one case the seizure frequency was transiently reduced by approximately 25%, in 3 cases no effects of taurine on the seizure frequency were observed during two weeks, while the seizures disappeared in five patients (König et al., 1977). Taurine treatment of children with epilepsy has been less successful. Oral administration of taurine from 0.05 to 0.3 g/kg daily to 25 children with intractable epilepsy aged from 4 months to 12 years diminished the seizure frequency by more than 50% in only one case, by less than 50% in four cases, and had no effect in 18 cases. Additionally, four patients exhibited side-effects of drowsiness and ataxia (Fukuyama and Ochiai, 1982).

On the average, about one third of human patients have shown significant alleviation of seizures under taurine medication (Oja and Kontro, 1983b). However, the efficacy of taurine tends to vanish with time, possibly as it does not readily penetrate into the brain and the body adapts itself to the increased taurine supply by enhancing taurine excretion into the urine. Taurine derivatives have also been tested as antiepileptic drugs, but so far none has been added to the arsenal of antiepileptic drugs in human patients (Oja and Saransaari, 2013b).

As stated above, taurine is the most abundant amino acid in the retina. It appears to be essential for normal vision and taurine deficiencies are linked with retinal degeneration (Lima, 1999; Gaucher et al., 2012). In cats, where taurine is an essential amino acid, taurine deficiency leads to retinal degeneration and eventual blindness along with low retinal and plasma concentrations of taurine (Hayes et al., 1975; Knopf et al., 1978). It is also thought that abnormalities in taurine metabolism might be associated with retinitis pigmentosa in humans (Airaksinen et al., 1980b; Kendler, 1989). It is assumed that taurine deficiency induced by the antiepileptic drug vigabatrin, which is widely used in the treatment of patients with epilepsy, is a
cause of retinal phototoxicity (Jammoul et al., 2009). In neonatal rats vigabatrin has induced taurine deficiency, cone photoreceptor damage, disorganization of the photoreceptor layer, gliosis and retinal ganglion cell loss. A daily taurine injection at a dose of 420 mg/kg restored blood plasma taurine level to the control value and partially prevented these retinal lesions and in particular the retinal ganglion cell loss (Jammoul et al., 2010). These findings underline the possible benefits of taurine supplementation for vigabatrin-treated epilepsy patients (Jammoul et al., 2009, 2010).

Taurine has beneficial effects against diabetes mellitus and its complications (Ito et al., 2012b). It has been shown that the taurine deficiency observed in diabetic patients (Franconi et al., 1995; De Luca et al., 2001) might be due to a decrease in both intestinal absorption of taurine and its renal reabsorption as well as an enhancement in urinary taurine clearance and fractional excretion (Merheb et al., 2007). In insulin-dependent diabetes both plasma and platelet taurine levels are decreased, but this is corrected by oral taurine supplementation (Franconi et al., 1995). In in vitro experiments, taurine has reduced platelet aggregation in diabetic patients in a dose-dependent manner, whereas 10 mmol/kg of taurine did not modify aggregation in healthy subjects (Franconi et al., 1995). In rats, taurine administration attenuates the increase in serum glucose and insulin levels after glucose loading. Also a 50 % increase in glycogen synthesis in the liver has been observed (Kulakowski and Maturo, 1984). Supplementation with 5 % taurine has reduced the mortality rate in diabetic rats and markedly prolonged their survival time (Franconi et al., 2003). It is thought that taurine exerts its effects on glucose homeostasis through two mechanisms; by influencing β-cell insulin secretion and by interfering with the insulin signalling pathway and post-receptor events (De la Puerta et al., 2010).

Taurine can be used in medicine not only as a drug, but also as a marker. For example, female patients with breast cancer have a markedly lower serum taurine level compared to patients from control or high-risk groups. Assessment of the taurine level in the sera of patients with a high risk of breast cancer seems to be a particularly useful tool in the early diagnosis of any malignant changes in the breast (El Agouza et al., 2011).

Despite the large body of data clearly showing the beneficial effects of taurine administration (described above), there are also a few publications
describing possible adverse effects of taurine application. For instance, there is some evidence that patients with psoriasis display extension of cutaneous lesions and an increased scale loss after 2 g taurine intake (Roe, 1966). In addition, taurine supplementation to rats in late gestation results in accelerated postnatal growth, this being associated with adult obesity and insulin resistance in the offspring (Hultman et al., 2007). In kainic acid-induced seizure models in mice (El Idrissi et al., 2003) and rats (Eppler et al., 1999) it has been shown that chronic administration of taurine in drinking water results in an increased susceptibility to seizures and a decreased latency for clonic seizures. These findings suggest the possibility that excessive consumption of popular energy drinks fortified with taurine could be a reason for seizures in some susceptible young individuals (Iyadurai and Chung, 2007; Calabró et al., 2012). Furthermore, it has recently been shown that intracerebroventricular administration of taurine impairs learning and memory in rats (Ito et al., 2012a). Although it is still generally believed that taurine supplementation at high doses is safe for humans (van de Poll et al., 2006), a healthy adult person is recommended not to take taurine at more than 3 g/d (Shao and Hathcock, 2008). Even though much higher taurine doses (up to 6 - 10 g/d) have been used in patients without any adverse effect (Durelli et al., 1983; Azuma et al., 1985), available clinical trial data published for humans are not sufficient for a confident conclusion as to the long-term safety of taurine at doses exceeding 3 g/d (Shao and Hathcock, 2008).

6.2 Cell Death

6.2.1 Cell Death Modalities

According to Clarke and Clarke (1996, 2012) the concept of cell death was first introduced in 1842 by Carl Vogt, who focused on its occurrence in the developmental metamorphosis of the midwife toad. Many researchers reported similar observations on cell death during the second half of the 19th century, especially from studies on the metamorphosis of insects and amphibians (Clarke and Clarke, 1996, 2012). In 1964 an article entitled “Programmed Cell Death” was published in which the phenomenon "programmed cell death" was described, i.e., a
sequence of controlled (and thereby implicitly genetic) steps towards the destruction of cells (Lockshin and Williams, 1964; Lockshin and Zakeri, 2001). Between 1962 and 1964 histological studies of ischemic liver injury allowed John F. Kerr to distinguish two types of cell death: classical necrosis and a process involving conversion of scattered cells into small round masses of cytoplasm which often contained specks of condensed nuclear chromatin, first called shrinkage necrosis (Kerr, 1965, 1971, 2002) and later renamed apoptosis. The term apoptosis was coined in 1972 by John F.R. Kerr and co-workers (1972) to designate the new form of cell death in the article "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics". The word "apoptosis" means in Greek the "dropping off" or "falling off" of petals from flowers or leaves from trees. Apoptosis initially aroused little attention, but during the last decade of the 20th century interest for the process has grown rapidly (Melino et al., 2001; Lawen, 2003) and is still growing.

Initially the concept of cell death modalities distinguished two types of death: one is regulated – apoptosis - the other accidental – necrosis. Two terms, apoptosis and programmed cell death, were often used as synonyms. With increasing knowledge of programmed cell death several attempts were made to classify cell death on morphological characteristics (Schweichel and Merker, 1973; Levin et al., 1999; Kroemer et al., 2005, 2009). It was long assumed that the same morphological pictures represent the activation of identical or at least similar lethal signal cascades. Only recently has it become clearer that apparently similar morphotypes of cell death often involve a great degree of functional, biochemical and immunological heterogeneity (Galluzzi et al., 2007; Green et al., 2009). Based on substantial progress in the biochemical and genetic exploration of cell death, the Nomenclature Committee on Cell Death 2012 recommended the use of molecular definitions of cell death subroutines (Galluzzi et al., 2012). According to this proposal the functional classification of cell death modalities applies to both in vitro and in vivo settings and includes extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophes. The Committee also discussed tentative definitions of other cell death modalities, among them anoikis, entosis, parthanatos, pyroptosis, netosis and cornification. Despite the diversity of cell death modalities discovered during recent years, it seems that the most prominent of them - necrosis, autophagic cell
death (autophagy) and apoptosis - are more popular among researchers and are being intensively studied (Ouyang et al., 2012; Nikoletopoulou et al., 2013). These types of cell death (autophagy, apoptosis and necrosis) are responsible for neuronal loss in the brain, occurring in different pathological conditions including cancer, neurotrauma, cerebral ischemia or intoxication (Orrenius et al., 2011; Ouyang et al., 2012; Pais et al., 2013).

Until recently, necrosis was considered an “accidental” mode of cell death occurring in an uncontrolled manner. However, with the discovery of the key mediators of necrotic death, for example receptor interacting protein (RIP) kinases and poly(ADP-ribose) polymerase-1 (PARP1), it is becoming clear that necrosis is as well controlled and programmed as for example caspase-dependent apoptosis (Festjens et al., 2006; Golstein and Kroemer, 2007; McCall, 2010; Galluzzi et al., 2012; Ouyang et al., 2012). The main morphological and physiological distinction between necrosis and apoptosis or autophagy is in the disruption of cell membrane integrity, when intracellular materials are released into the extracellular space, leading to inflammatory responses by immune cells (Majno and Joris, 1995; Elmore, 2007; Ulukaya et al., 2011; Ouyang et al., 2012). Although this inflammation is not a primary cause of such diseases as atherosclerosis, obesity, cancer, chronic obstructive pulmonary disease, asthma, inflammatory bowel disease, neurodegenerative disease, multiple sclerosis or rheumatoid arthritis, it contributes significantly to their pathogenesis (Nathan and Ding, 2010).

Autophagy is an evolutionarily conserved type of programmed cell death. It is a catabolic process which begins with the formation of double-membrane-bound structures (autophagosomes) surrounding cytoplasmic macromolecules and organelles intended for recycling (Mizushima, 2007; Huett et al., 2010; Ouyang et al., 2012). Autophagy plays a crucial pro-survival role in cell homeostasis during periods of starvation or stress due to growth factor deprivation (Mizushima, 2007; He and Klionsky, 2009) as well as in a growing number of pathological conditions, including cancer, myopathies and neurodegenerative disorders (Mariño and López-Otín, 2004). The implication of autophagy in many pathological processes and the possibility to regulate it by drugs make it attractive for clinical use. However, current therapeutic targeting of autophagy in human disease is limited by an incomplete understanding of how the process contributes to pathogenesis, the lack
of specificity of compounds which can influence autophagy, and the limited availability of candidate therapeutics with clinical efficacy (Choi et al., 2013).

As this present work focuses on the neuroprotective role of taurine in apoptosis regulation, apoptotic programmed cell death will be considered in greater detail.

6.2.2 Apoptosis

Apoptosis is a genetically programmed, morphologically distinct form of cell death which can be triggered by a variety of physiological and pathological stimuli. It is a normal component in the development and health of multicellular organisms (Hotchkiss et al., 2009). Every second, almost one million cells are eliminated in the body by apoptosis and replaced with new cells (Ulukaya et al., 2011). When cells die via apoptosis they do so in a controlled and strictly regulated manner. A dying cell plays an active role in its own death (hence apoptosis is often referred to as cell suicide) (Kondo, 1988; Majno and Joris, 1995).

Apoptosis is associated with a distinct set of biochemical and morphological changes which involve the cytoplasm, nucleus and cytoplasmic membrane (Häcker, 2000; Hengartner, 2000). Early in the process the cells round up, lose contact with their neighbors and shrink (Kerr et al., 1972; Wyllie et al., 1980). Thus apoptosis was first called shrinkage necrosis (Kerr, 1965, 1971). In the nucleus, chromatin condenses and aggregates into dense compact masses (Kerr et al., 1972; Majno and Joris, 1995). It is also fragmented internucleosomally by endonucleases (Arends et al., 1990; Zhivotovsky et al., 1994; Parrish and Xue, 2006). The progression of condensation is accompanied by the convolution of the nuclear and cell outlines, followed by disintegration of the nucleus into discrete fragments, which are surrounded by a double-layered envelope, and by budding of the whole cell to produce membrane-bounded apoptotic bodies, many of which contain nuclear fragments. The cytoplasmic organelles of the newly formed apoptotic bodies remain well preserved (Kerr et al., 1972, 1994).

The membrane of apoptotic cells remains intact and shows irregular buds known as membrane blebs (Majno and Joris, 1995; Saraste and Pulkki, 2000). It is assumed that hydrolysis of sphingomyelins embedded in the cell membrane and
cholesterol efflux are responsible for this development (Tepper et al., 2000). The plasma membrane is modified by externalization of phosphatidylserine, which enables the recognition of apoptotic bodies by phagocytic cells (Schroit et al., 1985; Fadok et al., 1992; Martin et al., 1995; Wickman et al., 2012). The phagocytosed apoptotic bodies are digested by a lysosomal mechanism (Bursch et al., 1985; Kerr et al., 1994) and the apoptotic cell finally disappears without a trace. Hence apoptosis does not usually lead to inflammation and can be regarded as a physiological form of cell death (Ulukaya et al., 2011).

6.2.3 Caspases in Apoptosis

Apoptosis is an evolutionarily conserved form of cell suicide, which requires specialized machinery for cellular self-destruction. The central component in this machinery is a proteolytic system, involving special enzymes called caspases (Thornberry and Lazebnik, 1998; Boatright and Salvesen, 2003). The caspases are a family of aspartate-specific cysteine proteases which are involved in various pathways of programmed cell death and play a crucial role as initiators and effectors of apoptosis (Budihardjo et al., 1999; Earnshaw et al., 1999; Pop and Salvesen, 2009).

The caspases are synthesized as inactive pro-forms or zymogens. These zymogens can cleave to form active enzymes following the induction of apoptosis. According to the timing of their activation and their function during apoptosis, the caspases can be defined into two sub-groups as upstream or initiator (caspase-2, -8, -9 and -10) and downstream or effector (caspase-3, -6 and -7) caspases. The initiator caspases become activated mainly through two pathways, the extrinsic or receptor-mediated and the intrinsic or mitochondrial, which involve the assembly of constitutive factors into the large protein complexes required for the process of initiator caspase activation. The activated initiator caspases cleave and activate effector caspases. These are responsible for the cleavage of numerous proteins involved in the signalling pathways and the key structural components of the cytoskeleton and nucleus (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999). For instance, caspases cleave such important substrates as PARP (poly(ADP-Ribose) polymerase), which is an enzyme involved in DNA repair, DNA-PK (a
DNA-dependent protein kinase), pRb (the product of the retinoblastoma gene, which is responsible for cell cycle arrest), lamins (structural proteins located at the nucleus membrane), NuMA (nuclear mitotic apparatus protein), fodrin and actin (structural proteins involved in the cytoskeleton), Mdm2 (a protein inactivating tumor suppressor protein p53) and cyclin A2 (which is involved as a promoter in the cell cycle) (Ulukaya et al., 2011). Caspases activate the caspase-activated DNase, which executes endonucleolytic chromosome degradation. Finally, all these events lead to the typical morphological changes observed in cells undergoing apoptosis (Enari et al., 1998; Earnshaw et al., 1999; Budihardjo et al., 1999; Slee et al., 2001).

Although caspases play a crucial role in apoptosis induction and execution (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999; Ulukaya et al., 2011), there is growing evidence to indicate that caspases performs other functions in the cell which are not connected to cell death, for example proliferation, differentiation, cell shape and cell migration (Miura, 2013). It has previously been shown that other apoptotic proteins like p53, p21(Waf1/Cip1) and Bcl-2 proteins, besides affecting the cell cycle, tumor suppression and apoptosis, may also act as modulators of the neurosecretory activity of hypothalamic neurons (Chernigovskaya et al., 2005).

6.2.4 Pathways of Apoptosis

The caspases play an important role in the induction and execution of apoptosis. Now the two main pathways of apoptosis are considered, each of them including activation of one of the initiator caspases.

6.2.4.1 Extrinsic or Receptor-Mediated Apoptosis

One major pathway for the induction of apoptosis is the receptor-mediated or extrinsic pathway (Fig. 2). The receptors triggering this pathway are located in the plasma membrane of the cell which is to undergo apoptosis (Kruidering and Evan, 2000; Lavrik and Krammer, 2012). They are activated by extracellular ligands. Typical death receptors are the Fas (also called Apo-1 or CD95) and the tumor-necrosis factor receptor (TNF-R)-1. They belong to the TNF-R family and contain a cytosolic death domain (DD). In the Fas signaling, the FasL binds to Fas, leading to
receptor trimerization. Adaptor proteins via their DDs (Fas-associated death domain protein, FADD) then bind to the cytosolic death domains (DD) of Fas. In addition, the FADD contains a death effector domain (DED), to which the DED of pro-caspase-8 can bind. The complex of Fas, FasL, FADD and pro-caspase-8 is called DISC. The pro-caspase-8 molecules are brought into close proximity in the DISC, and can thus transactivate one another. Active caspase-8 consists of a tetramer with two large and small subunits (Crowder and El-Deiry, 2012). The active caspase-8 can then directly cleave the caspase-3 or other executioner caspases, this leading eventually to the apoptotic outcome (Budihardjo et al., 1999; Galluzzi et al., 2012). The caspase-8 also can cleave the BH3-only protein Bid. The resulting truncated Bid (tBid) then moves to the mitochondria and induces cytochrome C release, leading to the activation of caspase-9 and caspase-3 (Li et al., 1998; Scaffidi et al., 1998; Kaufmann et al., 2012).

6.2.4.2 Intrinsic or Mitochondria-Mediated Apoptosis

In response to multiple intracellular stress conditions (e.g., DNA damage, cytosolic Ca^{2+} overload, oxidative stress), mitochondrial membranes became disrupted and form permeability transition pores (Fig. 2). Cytochrome C is released through these pores from the mitochondrial intermembrane spaces into the cytosol, where it binds to the apoptotic protease activating factor-1 (Apaf-1). This leads to the recruitment of pro-caspase-9 into a multi-protein complex with cytochrome C and Apaf-1, called the apoptosome, which contains seven Apaf-1, seven cytochrome C, seven (d)ATP and seven pro-caspase-9 molecules (Purging-Koch and McLendon, 2000; Riedl and Salvesen, 2007). The formation of the apoptosome leads to the activation of initiator caspase-9 (Hu et al., 1998, 1999; Jiang and Wang, 2000; Würstle et al., 2012) and activates other caspases in a cascade (Slee et al., 1999). This cascade eventually leads to the activation of effector caspases such as caspase-3 and caspase-6 and finally destroys the cell.

In addition, AIF (apoptosis-inducing factor) is released from the mitochondria (Candé et al., 2002). This factor causes degradation of DNA by activating some nucleases as yet not defined, the morphological appearance,
however, being different from that of typical apoptosis, which is characterized by irregular and peripheral nucleus fragments.

### Apoptotic signalling pathways

![Diagram of apoptotic signalling pathways]

Fig. 2. Simplified scheme of two main apoptotic pathways.

#### 6.2.5 Significance of Apoptosis in Health and Disease

Apoptosis occurs during the normal development of multicellular organisms and continues throughout adult life (Kerr et al., 1994). The combination of apoptosis and cell proliferation is responsible for shaping tissues and organs in developing embryos. For example, apoptosis is responsible for the regression of the tadpole tail which takes place during metamorphosis into a frog (Kerr et al., 1974) and for the elimination of cells located in between the toes which allows their separation (Penaloza et al., 2006). In the adult organism apoptosis is needed to maintain the
delicate balance between the increase (by differentiation from precursors and by proliferation) and the decrease (by further differentiation and cell death) in the number of cells in tissues. If mitosis were to proceed without cell death, an 80-year-old person could have 2 tons of bone marrow and lymph nodes and a gut 16 km long (Melino, 2001).

Apoptosis also plays an important role in regulation of the immune system. T lymphocytes are immune cells responsible for destroying infected or damaged cells in the body. They mature in the thymus, but before they can enter the bloodstream they are tested to ensure that they are effective against foreign antigens and not reactive against normal, healthy cells. Ineffective or self-reactive T-cells are removed through the induction of apoptosis (Ekert and Vaux, 1997). Any significant abnormality in apoptosis regulation leads to a disease (Rudin and Thompson, 1997; Ulukaya et al., 2011). For example, cancer is often characterized by a dearth of apoptosis (Thompson et al., 1992). There is a considerable body of evidence that alterations in apoptosis are involved in breast (Bachmann et al., 2007; Janes, 2011; Tawfik et al., 2012), lung (Han et al., 2002; Viard-Leveugle et al., 2003), kidney (Gobé et al., 2002) and CNS (Hervouet et al., 2010; Goldberg et al., 2011; Stegh and DePinho, 2011) cancer, for example. Cancer cells typically evince a number of mutations which allow them to ignore normal cellular signals regulating their growth and become more proliferative than other cells (Zielinski et al., 2013). Under normal circumstances the damaged cells will undergo apoptosis, but in the case of cancer cells mutations may prevent this (Wyllie, 1985; Isaacs, 1993). By reason of these mutations cancer cells also become resistant to radiation and chemical therapy (Rudin and Thompson, 1997; Igney and Krammer, 2002). Understanding how apoptosis is regulated in cancer is therefore one important problem in developing cancer treatment (Dive et al., 1992; Kerr et al., 1994; Zielinski et al., 2013).

Deficient apoptosis may be one reason for tumor development. However, dysregulation leading to excessive apoptosis is another side of the problem. For example, an increased rate of apoptosis in the adult nervous system is thought to account for the massive cell death and progressive loss of neurons appearing in neurodegenerative conditions like Parkinson’s, Alzheimer’s and Huntington's diseases (Su et al., 1994; Smale et al., 1995; Mochizuki et al., 1996; Nijhawan et al., 2000; Gil and Rego, 2008). It assumed that the therapeutic use of apoptosis modulators would have potential benefits in the treatment of such neurodegenerative
disorders (Deigner et al., 2000; Favaloro et al., 2012). Another example of a disease involving extensive apoptosis is heart failure (Das, 2007; Lee and Gustafsson, 2009; Fujita and Ishikawa, 2011). It has been shown in human patients (Olivetti et al., 1997; Akyürek et al., 2001) and in animal models (Sharov et al., 1996; Shizukuda et al., 2005) that apoptosis is one of the major mechanisms causing cardiomyocyte loss in the failing heart. It seems that selective inhibition of the proteolytic functions of caspase-3, the major executor of apoptosis, may represent an attractive approach to attenuate or reverse heart failure (Reeve et al., 2005; Yang et al., 2013).

The important role of apoptosis in wound healing has also been demonstrated. Wound healing involves a series of rapid increases in specific cell populations which prepare the wound for repair, deposit new matrices and finally heal the wound. Upon completing their tasks, these specific cell types must be eliminated from the wound prior to progression to the next phase of healing. Apoptosis allows the elimination of entire cell populations without tissue damage or inflammatory response, and dysregulation of apoptosis can lead to pathologic forms of healing such as excessive scarring and fibrosis (Greenhalgh, 1998; Rai et al., 2005).

During the past 50 years intensive research has been done in the field of cell death, going far to clarify many aspects of this fundamental process and its involvement in a large number of different diseases. The progress in investigation of the mechanisms of apoptosis and other forms of cell death made in recent years gives hope of successful application of modern approaches based on cell death mechanisms in clinical practice in the treatment of a wide range of pathologies (Ulukaya et al., 2011; Favaloro et al., 2012). However, many problems remain to be solved before the adoption of these methods in medicine; for example, how to activate an alternative death pathway when one is pharmaceutically blocked (in diseases where survival of the target cell is the final goal), and/or how to protect "innocent bystanders" from unwanted death when attempting to kill pathological cells as in anti-tumor therapies (Favaloro et al., 2012).
6.2.6 Apoptosis in Ischemia

Ischemic injury causes severe neurodegeneration and consequently a loss of normal brain functions (Schwartz et al., 1998; Block, 1999; Lipton, 1999) and is the leading cause of acquired adult disability in the USA (Lloyd-Jones et al., 2010). Ischemic injury is caused by the loss of blood flow to the brain, usually in consequence of an embolism. The mechanisms underlying neuronal degeneration and the potential neuroprotective effects of some pharmacological treatments have been actively studied. As shown in various animal models (Garcia et al., 1995; Lipton, 1999; Liou et al., 2003), a decrease in perfusion determines both apoptotic and necrotic neuronal death in the affected region (core) due to energy depletion (Yuan, 2009; Downes and Crack, 2010). In the ischemic core it is difficult, even impossible, to prevent cell death. Around this area of tissues, which is irreversibly lost, there is an area of partially damaged tissue known as the penumbra which triggers local inflammation. Some evidence demonstrates that inflammation is a crucial event in the progression of ischemic brain damage (Takadera et al., 2004; Adibhatla and Hatcher, 2006). In the penumbra dying cells display morphological manifestations of apoptosis (Linnik et al., 1993; MacManus et al., 1993) and with appropriate treatment they may be saved (Goto et al., 1990; Linnik et al., 1995; Martin-Villalba et al., 2001). Evidence is also mounting to indicate a major role of caspases in ischemia-mediated cell death (Thornberry and Lazebnik, 1998; Plesnila et al., 2001; Love, 2003). The caspases begin to be expressed at high levels and become activated under post-ischemic conditions (Ferrer and Planas, 2003).

An important factor in ischemic brain damage is activation of the receptor-mediated apoptotic pathway, as proved by increased levels of Fas and FasL during brain ischemia (Tian et al., 2009) and by the finding that TNF deletion in mice protects the brain from ischemic damage (Martin-Villalba et al., 2001). It has been proposed that the activation of apoptosis proceeds via different and specific pathways in the core and in the penumbra area of the brain infarction. Thus in neurons in the core area the first apoptotic events are mediated by ligand binding to specific death receptors, leading to caspase-8 activation, whereas in the penumbra area, where mitochondria provide a residual energy supply, neuronal death is in contrast induced through the mitochondrial pathway (Benchoua et al., 2001).
The involvement of caspases in mediating ischemic neuronal cell death is also proved by that fact that a genetic knockout or pharmacological block of caspases seems to be effective in reducing cell damage in both in vivo and in vitro models of ischemia (Le et al., 2002).

### 6.2.7 Apoptosis in Fetal Alcohol Syndrome

Alcohol consumption during pregnancy is a worldwide problem appearing on all continents and makes no exception for rich or poor countries (May and Gossage, 2001; Autti-Rämö et al., 2006; May et al., 2006, 2007; Kristjanson et al., 2007; Popova et al., 2012; Centers for Disease Control and Prevention, 2012; O'Leary et al., 2013). It is well established that alcohol consumption during pregnancy is harmful to the fetus, especially to its developing nervous system (Warren and Bast, 1988; Krulewitch, 2005; Dalen et al., 2009). This notwithstanding, in the United States about 11% of pregnant women continue to consume alcohol during their pregnancies (The NSDUH Report 2008). In Finland, the corresponding percentage is 3-6% (Autti-Rämö, 1997; Pajulo et al., 2001). The rate of alcohol consumption per person per year in Russia is among the highest in the world (World Health Organization, 2005, 2011). The estimated percentage of Russian women consuming alcohol during their pregnancies is also the highest, varying between 43-60% (Chambers et al., 2006; Kristjanson et al., 2007). Depending on the frequency of drinking, the amount of alcohol consumed and the stage 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 for instance growth retardation, structural brain abnormalities, behavior and cognitive problems, and learning difficulties (Clarren et al., 1978; Famy et al., 1998; Chudley et al., 2005; Spadoni et al., 2007). The prevalence of children affected by prenatal alcohol exposure (FAS and alcohol-related birth defects) remains too high, being about 1 per cent of all children born in the United States, even though not all babies born of alcohol-drinking women have symptoms of alcohol-related disorders (May and Gossage, 2001). It has been estimated that in Finland 80-100 newborns yearly suffer from FAS (Autti-Rämö, 1997; Pajulo et al., 2001), the most serious form of deficit. The actual prevalence of
FAS in Russia is unknown (Shilko et al., 2010). In screening evaluations of fetal alcohol spectrum disorders in all 234 baby home residents in the Murmansk region of Russia, 13% of children yielded high scores suggesting FAS and 45% evinced intermediate phenotypic expression of fetal alcohol spectrum disorders (Miller et al., 2006).

The human fetal brain is particularly sensitive to the adverse effects of alcohol during the last trimester of pregnancy, the period of synaptogenesis, also known as the brain growth spurt period. In rodents, the same period of increased sensibility to ethanol occurs during the early postnatal period (Dobbin and Sands, 1979; Rice and Barone, 2000). It has been well documented in rodents in vivo (Ikonomidou et al., 2000; Olney et al., 2000, 2002a, b; Dikranian et al., 2005; Kumral et al., 2005; Young and Olney, 2006) and humans in vitro (Hao et al., 2003) that alcohol induces massive death by apoptosis of neurons in the developing brain. Such widespread cell death may account for the morphological and functional disorders and retardation in learning of children with FAS (Hao et al., 2003). It has been suggested that ethanol, which has both N-methyl-D-aspartate (NMDA) antagonist and GABA<sub>A</sub> agonist properties, triggers apoptotic neurodegeneration in the developing brain by a dual mechanism – transient blockade of NMDA receptors and excessive activation of GABA<sub>A</sub> receptors (Olney et al., 2000; Ikonomidou et al., 2001). In addition, it has been shown that ethanol induces endoplasmic reticulum (ER) stress in the developing brain, although the linkage between ER stress and neurodegeneration remains to be established (Ke et al., 2011).

The important role of caspase-3 activation in the execution of ethanol-induced apoptosis has been shown (Olney et al., 2002a; Sari, 2009; Sari et al., 2009). It was already described above that caspase-3 become activated via both extrinsic and intrinsic apoptotic pathways (Boatright and Salvesen, 2003). A further investigation of the molecular mechanisms involved in the regulation of ethanol-induced apoptosis carried out in Bax-knock-out mice revealed that ethanol-induced apoptosis in the developing brain is a Bax-dependent process which requires translocation of the Bax protein from cytosol to mitochondria, disruption of mitochondrial membranes, release of cytochrome C and activation of caspase-3 (Young et al., 2003; Nowoslawski et al., 2005). All these events, together with the absence of caspase-8 activation, indicate that alcohol induces apoptosis in the developing brain via intrinsic (mitochondria-mediated) apoptotic pathway (Young
et al., 2003; Nowoslawski et al., 2005; Balaszczuk et al., 2011), whereas ischemia, for example, induces apoptosis through both extrinsic and intrinsic pathways (Benchoua et al., 2001). It has also been shown that interaction of proapoptotic protein Bax with the proteins of the mitochondrial permeability transition pore, among them the voltage-dependent anion channel proteins of the outer mitochondrial membrane and the adenine nucleotide translocator proteins of the inner membrane, are critical for ethanol neurotoxicity in the neonatal rat cerebellum, leading to loss of the mitochondrial membrane potential, release of mitochondrial contents such as cytochrome C, and initiation of the apoptosis cascade. An inhibition of such interactions between Bax and proteins of the mitochondrial permeability transition pore in vitro sufficed to block ethanol neurotoxicity (Heaton et al., 2013). Prevention of ethanol-induced apoptosis can save a huge number of neurons and significantly reduce the harmful consequences of alcohol intoxication. Neuroprotective effects of ADNF-9 (a peptide derived from the activity-dependent neurotrophic factor) have been demonstrated in a mouse model of fetal alcohol exposure (Sari et al., 2012). Thus, ADNF-9 administration prevents alcohol-induced reduction in fetal brain weight, alcohol-induced reduction in the level of the antiapoptotic Bcl-2 protein and also alcohol-induced cell death.
7. Aims of the Study

The aim of the present study was to characterize the neuroprotective functions of taurine under different pathological conditions.

The specific aims were as follows:

1. to study the influence of taurine on the expression of pro-apoptotic signalling proteins caspase-8 and caspase-9 in the supraoptic (SON) and paraventricular (PVN) nuclei of the mouse hypothalamus under ischemia-mimicking conditions in vitro;

2. to assess the neuroprotective action of taurine against apoptosis induced by acute alcohol administration in three different neuronal layers of the developing cerebellum, external granular layer, Purkinje cell layer and internal granular layer in mice in vivo;

3. to describe a new phenomenon comprising the combined toxicity of taurine and ethanol in mice in vivo and to establish its possible mechanisms.
8. Materials and Methods

8.1 Animals (I-V)

Adult NMRI (Naval Medical Research Institute) mice for experiments and breeding were purchased from Harlan, the Netherlands. In the experiments male and female mice of five age groups were used: 4-day-old (day of birth is day 0) (III), 7-day-old (II-V), young adult (3-month-old) (I), adult (5-6 month-old) (V) and old (12-13-month-old) (V). In the experiments altogether 259 mice were used.

8.2 Ethical Considerations

The experiments on animals were carried out in accordance with the European Community Council Directive 86/609/EEC. All efforts were made to reduce the number of animals and to minimize their suffering. Permission for the experiments was first granted by the Animal Experiment Committee of Tampere University, numbers 996, 1013 and 1045, and then by the National Animal Experiment Board ESLH-2008-03399/Ym-23 and ESAVI-2010-08483/Ym-23.

8.3 Experimental Models

8.3.1 Ischemia-Induced Apoptosis In Vitro (I)

Young adult (3-month-old) male NMRI mice were decapitated and their brains rapidly excised and placed in ice-cold oxygenated (95 % O₂, 5 % CO₂) artificial cerebral spinal fluid (ACSF) containing (in mM) 126 NaCl, 5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 2.4 CaCl₂, 10 D-glucose, and 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (Hepes), pH 7.4. Slices
containing the SON and PVN (400-500 μm thick) were manually prepared and preincubated (two slices in each chamber) in 30 ml ACSF bubbled with 95 % O₂ and 5 % CO₂ in a shaking water bath at 37°C for 30 min. After the preincubation period the slices were divided into 3 experimental groups. Those in the first (“control”) group were incubated under the above-mentioned conditions until the end of experiments. The slices in the second (“ischemic”) group were incubated for 30 min in ACSF without glucose and bubbled with N₂ to mimic ischemic conditions. The third group (“ischemia + taurine”) were incubated for 30 min in ACSF with taurine (20 mM) without glucose and bubbled with N₂. This high taurine concentration was used as it has been shown that 20 mM of taurine most effectively protects tissue against apoptosis and cell loss (Eppler and Dawson, 2002; Takahashi et al., 2003; Takatani et al., 2004).

After the 30-min “ischemic” period, the medium was replaced every 30 min with fresh ACSF bubbled with 95 % O₂ and 5 % CO₂ to simulate a “reperfusion” period. In the “ischemia + taurine” group taurine was present during all periods of incubation. The slices were removed 180 min after the end of the “ischemic” period and fixed in 4 % paraformaldehyde in sodium phosphate buffer (PBS) (0.1 M, pH 7.4) overnight at 4°C.

8.3.2 Ethanol-Induced Apoptosis In Vivo (II-IV)

The neuroprotective action of taurine against ethanol-induced apoptosis was studied in three different layers of the cerebellar vermis, external granular cell layer (IV), Purkinje cell layer (III) and internal granular cell layer (II, III). The Purkinje cells were identified by their size, shape and specific localization in the cerebellar lobules. As no specific labeling was used to identify different types of neurons and glial cells in the external and internal granular cell layers of the cerebellum, these cells are referred to as external granular layer (EGL) and internal granular layer (IGL) cells. Since in developing mice the Purkinje cells are most sensitive to ethanol administration on P4 and EGL and IGL cells on P7, 4-day-old mice were used to study ethanol and taurine effects on the Purkinje cells and 7-day-old mice for these effects on EGL and IGL cells.
To reduce the possible influence of individual hereditary characteristics on experimental results the mice were divided 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). In brief, 20 % w/v ethanol solution in sterile saline was administered subcutaneously to the ethanol and ethanol+taurine groups at a total dose of 5 g/kg (2.5 g/kg at 1 h and 2.5 g/kg again at 3 h). These injections elevated the concentration of ethanol in the blood for 8 h at least to 40 mmol/l and led to significant and widespread apoptotic neurodegeneration in the developing brain (Ikonomidou et al., 2000) and cerebellum (Dikranian et al., 2005). The ethanol+taurine group also received two additional 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 one hour after the second ethanol injection. The control animals were given saline subcutaneously. Twelve (P4) and eight hours (P7) after the first ethanol injection the mice were killed by decapitation. Blood samples from each animal were collected separately in lithium-heparin tubes and centrifuged at 1750 rpm for 10 min to obtain plasma. The samples were kept frozen until HPLC analyses. The cerebella were rapidly excised and fixed in 4 % paraformaldehyde in phosphate-buffered saline for at least 3 days at 4°C. After routine histological processing they were embedded in paraffin and cut with a microtome into 5-μm thick mid-sagittal sections containing lobules I-X of the cerebellar vermis.

8.3.3 Combined Toxicity of Ethanol and Taurine In Vivo (V)

To assess the possible toxic effects of taurine and ethanol co-administration in different age groups we used developing (7-day-old), adult (5-6-month-old) and old (12-13-month-old) mice. The mice were divided into four experimental groups: control, ethanol-treated, taurine-treated and ethanol+taurine-treated. Ethanol (20 % w/v solution diluted in saline) and taurine (7 % w/v solution diluted in saline) were administered subcutaneously to 7-day-old mice and intraperitoneally to adult and old mice. The different doses of ethanol tested ranged from 0 to 12 g/kg and of taurine from 0 to 12 g/kg together with their combinations, the objective being to find a minimal 100 % lethal dose at each age. Taurine and ethanol were injected in
two half-doses: taurine at 0 h and 4 h, ethanol at 1 h and 3 h. The control animals were given saline injections equal to those given to the ethanol+taurine-treated group. The animals were monitored for 14 days to detect any signs of toxicity or lethality.

8.4 Conventional Histology (I-IV)

Paraformaldehyde-fixed tissue - brain slices from experiments with ischemia (I) and the whole cerebella from experiments with ethanol (II-IV) - were embedded in paraffin and cut with a microtome into 5 μm-thick frontal sections (brain) or mid-sagittal sections (cerebellum). The sections were mounted on glycerin-albumin-coated slides and dried overnight at 37ºС. Hematoxylin-eosin staining was used to visualize the general histoarchitecture of the slices after incubation in vitro. Deparaffinized sections were hydrated over the xylene and ethanol series to distilled water and stained with hematoxylin, washed, stained with eosin, washed again and dehydrated using ethanol and xylene for embedding.

8.5 Immunohistochemistry (I-IV)

The brain (I) and cerebellar (II-IV) 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 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) to reduce any nonspecific labeling. Thereafter, the specimens were incubated with primary antibody (diluted 1:200 in serum-blocking solution) in moist chambers overnight at 4ºC. We used the following primary antibody: polyclonal caspase-8 antibody (Caspase-8 (FLICE) Ab-4, Lab Vision Corp.) and polyclonal caspase-9 antibody (sc-8355, Santa Cruz Biotechnology, Inc.) in study I, and polyclonal activated caspase-3 antibody [cleaved caspase-3 (Asp 175) antibody, Cell Signaling Technology Inc.] in studies II-IV. On the following day the sections were washed with 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 immunoreactivity. The sections serving as negative controls were incubated without the primary antibody to rule out nonspecific staining. Finally, the sections with (III, IV) or without (I, II) additional counter-staining with hematoxylin-eosin were dehydrated and mounted.

8.6 Detection of Cell Death In Situ (II-IV)

DNA fragmentation is one of the widely accepted markers by which apoptotic cells are recognized (Sgonc and Gruber, 1998; Saraste and Pulkki, 2000; Stadelmann and Lassmann, 2000). 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, as H₂O₂ weakens terminal deoxynucleotidyl transferase activity (Migheli et al. 1995) and induces DNA breaks (Wijsman et al., 1993). The 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 room temperature and then counterstained with hematoxylin-eosin.

8.7 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 x 75 and x 750) using an image analysis system comprising an IBM PC, Nikon Microphot-FXA microscope, SensiCam digital camera (PCO Computer Optics GmbH), Image-Pro Plus (Media Cybernetics) to analyze caspase-8, caspase-9 and
activated caspase-3 immunoreactivity and TUNEL-staining. At least five sections cut from every animal at the same level of the hypothalamus (I) or of the cerebellar vermis (II-IV) were analyzed. The number of hypothalamic neurons from the SON and PVN labeled for caspase-8 and caspase-9 were calculated and expressed as mean ± SEM per slice (I). The number of activated caspase-3-IR or TUNEL-positive neurons from the internal (IGL) (II, III) and external (EGL) (IV) granular layer and from the Purkinje layer (III) of cerebellar lobules was counted in every slice and the area of the IGL, EGL or Purkinje layer in each lobule measured. The data are presented as the average number of labeled cells in the IGL, EGL or Purkinje layer per mm² for each experimental group.

8.8 Semi-Quantitative Analysis of Caspases

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) program was used for a semi-quantitative analysis of caspase-8 and caspase-9 expression in the histological sections of the SON and PVN of the hypothalamus (I) and of the activated caspase-3 expression in the cerebellar lobules (II). At least five sections cut from every animal at the same level of each hypothalamic nucleus or cerebellar lobules were analyzed. The sections were reviewed at 250-fold magnification under a light microscope. The relative optical density of DAB precipitates in the perikarya 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). Optical density reflecting the content of the proteins studied in neurons was calculated as the GL of an immunoreactive field of cell by subtracting the background GL. The optical density of the background was estimated in the same slice in the field of non-immunoreactive brain or cerebellar tissue.
8.9 High-Performance Liquid Chromatography (HPLC) (III)

The blood serum samples were kept frozen (–20°C) and thawed immediately prior to analysis. The concentration of taurine in the blood serum was measured using HPLC with fluorescent detection after precolumn derivatization with o-phthaldialdehyde (OPA) using the analysis equipment system of Shimadzu Scientific Instruments (Kyoto, Japan). The separation column was 4.6 x 250 mm Ultropac 8 Resin, lithium form (Farmacia, Denmark). Derivatization of taurine was performed with the ortho-phthaldialdehyde (OPA) reagent (0.2 % OPA, 0.1 % mercaptoethanol and 1 % ethanol in 1 M borate buffer, pH 10.4). Elution was done with lithium citrate buffers in the following order: (1) 0.2 M, pH 2.80, (2) 0.3 M, pH 3.00, (3) 0.5 M, pH 3.15, (4) 0.9 M, pH 3.50, and (5) 1.6 M, pH 3.30. The fluorescence of taurine derivatives was measured with an RF-10A detector using excitation and emission wavelengths set at 340 nm and 450 nm, respectively. The concentrations of taurine were finally estimated using a commercial amino acid mixture (Pickering, UK) as external standard and diamino-n-butyrate as internal standard.

8.10 Blood Glucose Determination (V)

A part of the adult and old mice were used for measurement of blood glucose levels. Deaths of mice used for the glucose assays were not included in calculation of lethality to avoid any possible influence on the rate of lethality arising from the procedure of blood collection and hemorrhage. Blood samples were taken from the tail vein of each animal at two time-points, in adult mice two hours before the first taurine injection to measure the baseline level of glucose and then 0.5 h after the last taurine injection, and in old mice likewise two hours before the first taurine injection and then 1 or 2 or 3 h after the last taurine injection to assess the effect of ethanol and taurine on blood glucose. This difference in time-points of blood sampling in adult and old mice was adopted to obtain samples maximally close to and immediately prior to death. At each time-point duplicate blood samples
(5 µl) were collected into HemoCue Glucose cuvettes and immediately analysed in a
HemoCue B-Glucose Analyzer (HemoCue AB, Ängelholm, Sweden).

8.11 Data Expression and Statistical Analysis

The levels of caspase-8 (I), caspase-9 (I) and activated caspase-3 (II)
expression in neurons were measured by optical density and expressed as mean ±
SEM. The number of caspase-8- and caspase-9-IR cells was expressed by mean ±
SEM (I). The numbers of activated caspase-3-IR and TUNEL-positive cells were
expressed as mean ± SEM (II) or as mean ± SD (III, IV). Each value for blood
taurine (III) and blood glucose (V) was expressed as the mean ± SD. Data on mouse
mortality were expressed in percentages (V).

Statistical significance was determined by Student’s t test (I, II, V) and one-
way analysis of variance (ANOVA) among the experimental groups (I-V). When
ANOVA showed a significant difference, the post hoc Bonferroni test (III, IV) or
Tukey-Kramer post hoc test (V) was applied to demonstrate the difference.
Differences were considered significant when the calculated p value was < 0.05 (I,
II, III, V) or at least < 0.01 (IV).
9. Results

9.1 Effect of Taurine on Ischemia-Induced Expression of Caspase-8 and Caspase-9 (I)

Hematoxylin-eosin-stained paraffin sections displayed the normal parenchymal architecture and cell morphology of the SON and PVN in all experimental groups. Immunoreactive (IR) caspase-8 and caspase-9 cells were observed in the SON and PVN in all experimental groups, but in the “ischemic” group their numbers were significantly increased \( p<0.05 \) in both hypothalamic nuclei. Moreover, ischemia led to upregulation of caspase-8 expression in individual neurons of both SON and PVN. All these changes indicate that ischemia led to the initiation of the two main apoptotic pathways in the SON and PVN of the hypothalamus.

After incubation with taurine, the number of caspase-8-IR cells in the PVN was significantly decreased \( p<0.05 \) when compared to PVN cells in the ischemic group. Taurine also reduced caspase-8 immunoreactivity in neurons of the SON and PVN under ischemic conditions. The presence of taurine likewise affected the expression of caspase-9, reducing the number of caspase-9-IR cells after ischemia in the SON and PVN, and the expression of caspase-9 in neurons of SON in comparison with the taurine-untreated “ischemic” group.

9.2 Effect of Taurine on Ethanol-Induced Expression of Activated Caspase-3 (II, III, IV)

Study of the neuroprotective effects of taurine in the developing cerebellum focused on three different layers, EGL, IGL and Purkinje cells. Activated caspase-3-immunoreactive cells in the EGL (IV) and IGL (II, III) were found in each cerebellar lobule in the saline-treated control mice on P7, evidencing the physiological cell death which normally occurs at this period of development. Only
a few activated caspase-3-IR Purkinje cells (III) were found in the saline-treated control mice on P4.

In the ethanol-treated pups on P4 a large number of activated caspase-3-IR Purkinje cells were discernible in all lobules, the highest amounts in lobules IX and X (III). In lobules I-II, III, IV-V, VI-VII and VIII the number of caspase-3-IR Purkinje cells was also significantly increased when compared to the control group (III). On P7 ethanol administration induced very little response to caspase-3 activation in the Purkinje cell layer, this hardly differing from the control group. However, ethanol treatment on P7 induced widespread activation of caspase-3 in the IGL (II, III) and EGL (IV). The number of activated caspase-3-IR IGL cells in each lobule was markedly greater than in the saline-treated pups (II, III). The highest immunoreactivity for activated caspase-3 was found in I-II, III and IV-V vermian lobules (III). In the EGL 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 (IV).

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 entirely abolish it in any lobule (II). In the ethanol+taurine-treated group on P4 the number of activated caspase-3-IR Purkinje cells in each lobule was approximately the same as in the pups treated with ethanol only and significantly higher than in the saline-treated control group (III). However, in contrast to the Purkinje cells at age P4, taurine treatment significantly reduced the number of activated caspase-3-IR cells in the IGL (II, III) and EGL (IV) on P7 in each. The numbers of rescued cells from caspase-3 activation were different in different lobules, varying from 34 % to 41 % for lobules VI, VII and X, and from 45 % to 57 % for lobules I-II, III, IV-V, VIII and IX in the IGL (III). In the EGL the taurine protective effect against ethanol-induced activation of caspase-3 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 (IV).

We also measured the activated caspase-3 content in individual immunoreactive neurons in the IGL on P7 to reveal whether taurine treatment affects the degree of caspase-3 expression inside the cell. Our data show that the level of activated caspase-3 in individual immunoreactive neurons was
approximately the same in all experimental groups in all cerebellar lobules (II). This finding, together with a fact that taurine treatment reduced the number of caspase-3-IR neurons, shows that taurine did not lower the activation of caspase-3 in all neurons, but totally abolished the activation of caspase-3 in one group of neurons while having no effect on the others.

### 9.3 Effects of Taurine on Ethanol-Induced Apoptosis

TUNEL-positive cells (cells with fragmented DNA) were considered here to be apoptotic cells. Again we focused on three different layers of the developing cerebellum (EGL, IGL and Purkinje cells) as in the estimation of caspase-3 expression. A few TUNEL-positive cells in the Purkinje cell layer on P4 (III) and in the IGL (II, III) and the EGL (IV) on P7 were detected in the saline-treated control groups, showing physiological cell death during normal development. The number of cells undergoing physiological cell death in the Purkinje layer (III) seems to be lower than in the IGL (III) or in the EGL (IV) and was in accord with our data on activated caspase-3 immunoreactivity.

Ethanol treatment significantly increased the number of apoptotic cells in the Purkinje cell layer of P4 mice. The largest number of TUNEL-positive Purkinje cells was found in lobules IX and X (III), although in the other lobules studied their number was also much greater than in the control group. At age P7, ethanol administration induced massive apoptosis in the IGL (III) and EGL (IV) in all vermian lobules, as indicated by the increased numbers of TUNEL-positive cells.

Taurine treatment did not alter the number of TUNEL-positive Purkinje cells on P4 in any lobule studied when compared to the pups treated with ethanol only (III). The number of apoptotic Purkinje cells remained much greater than in the saline-treated control group. In the ethanol+taurine group at age P7 the number of TUNEL-positive IGL cells was reduced in comparison to the ethanol group, the change being statistically significant in all other lobules except lobules VI and VII (III). Although taurine treatment reduced the number of TUNEL-positive IGL cells induced by acute alcohol administration, the number of these cells in the ethanol+taurine group remained much larger than in the control group. Taurine preservation of IGL cells from DNA fragmentation varied in the lobules from 40 %
(lobule I-II) to 68 % (lobule IX). Similar results were obtained from the EGL cells on P7 (IV), where taurine treatment protected from 43 % to 69 % of cells from dying if compared to the ethanol group. This effect was statistically significant (P<0.01) in lobules III, IV-V, VII, VIII, IX and X.

9.4 Dynamics of Taurine Blood Concentration Changes During the Experiments (III)

We measured the taurine concentration in the blood serum of 7-day-old mice during the experiments from 4 h onwards after the first ethanol injection (1 hour after the last taurine injection) at every hour for 12 h and then again at 24 h. In the saline-treated control group and in the ethanol group the taurine concentration remained the same at 4 h, 12 h and 24 h. It was 1.00 ± 0.42 mmol/l in the control group and 0.85 ± 0.24 mmol/l in the ethanol group. Two subcutaneous injections of taurine each at a dose of 1 g/kg (one at 0 h and second at 4 h) markedly increased the taurine level up to 13.36 ± 2.73 mmol/l at 4 h after the first ethanol injection. For four hours (4 h-7 h) the concentration in the blood was maintained at about the same high level of 13.20 ± 1.83 mmol/l, and then it started to decline gradually, almost reaching the control level by 12 h (1.59 ± 0.83 mmol/l).

9.5 Combined Toxicity of Taurine and Ethanol in 7-Day-Old Mice (V)

To reduce the possible influence of individual hereditary characteristics on experimental results the mice from each litter were divided into different experimental groups. Each group consisted of an equal number of males and females. As there was no difference in lethality between the genders, we show combined total data on lethality in both. Taurine at a total dose of 4 g/kg co-administered with ethanol at a total dose of 5 g/kg killed 58 % of 7-day-old mice. An increase in taurine dose to 6 g/kg administered to ethanol-treated mice induced 100 % mortality. Though we monitored the mice for 14 days after treatment to detect any signs of toxicity, they usually died during the first hours after the last
taurine injection – about 60 % in the time frame between 0.5-1 h, others during the next hour (unpublished data). All mice exposed to ethanol or taurine alone and mice treated with 2 g/kg taurine co-administered with 5 g/kg ethanol survived.

9.6 Combined Toxicity of Taurine and Ethanol in Adult Mice (V)

In the first set of experiments on adult (5-6-month-old) mice we established that a total ethanol dose of 8 g/kg administered in two half-doses with a 2-hour interval was the maximal dose not lethal for mice of this age. Co-administration of taurine at a dose of 8 g/kg with ethanol (8 g/kg) killed 50 % of the adult mice. One hundred % lethality was observed at a taurine dose of 10 g/kg administered to ethanol-treated mice (V). All died during one hour after the last taurine injection (unpublished data). All mice treated with taurine or ethanol alone at the doses used survived for the 14 days of observation. All those treated with 6 g/kg taurine co-administered with 8 g/kg ethanol likewise survived.

9.7 Combined Toxicity of Taurine and Ethanol in Old Mice (V)

In the preliminary experiments on old (12-13-month-old) mice we found a total ethanol dose of 6 g/kg administered in two half-doses with a 2-hour interval to be the maximal dose not lethal for mice of this age. Testing different taurine doses co-administered with 6 g/kg of ethanol we found taurine at a dose of 4 g/kg administered to the ethanol-exposed mice to kill 22 % of treated mice. Taurine at a total dose of 6 g/kg combined with 6 g/kg ethanol resulted in 69 % mortality. At these highest doses of ethanol and taurine old mice died within a time frame from 2.5 h to 12 h after the last taurine administration (unpublished data). All mice treated with taurine or ethanol alone survived the whole 14-day period of observation.
9.8 Changes in Blood Glucose Level after Taurine and Ethanol Co-Administration in Adult Mice (V)

Adult mice treated with 10 g/kg taurine combined with 8 g/kg ethanol usually died within 1 h after the last taurine injection. To measure the blood glucose level maximally close to death we assayed it 0.5 h after the last taurine injection. Two hours before the treatment we measured the basic level of glucose. It was similar in all experimental groups (8.5 ± 0.9 mmol/l), with individual variations from 7.5 to 11.1 mmol/l. Co-administration of taurine and ethanol significantly reduced (P<0.05) the glucose level from 9.3 ± 1.6 mmol/l to 5.6 ± 0.7 mmol/l. Ethanol alone tended to increase blood glucose, but the difference from 8.6 ± 0.2 mmol/l to 12.3 ± 2.1 mmol/l, was not statistically significant by reason of wide variation. Taurine alone did not alter the blood glucose level.

9.9 Changes in Blood Glucose Level after Taurine and Ethanol Co-Administration in Old Mice (V)

Old mice treated with 6 g/kg of taurine and 6 g/kg of ethanol died within 3-4 hours after the last taurine injection. We measured blood glucose at 1, 2 and 3 hours after the last taurine injection to monitor changes in blood glucose prior to death. Two hours before the treatment the basic level of glucose was similar in all experimental groups (8.5 ± 1.2 mmol/l), with individual variations ranging between 6.9 and 11.2 mmol/l. Taurine and ethanol co-administration significantly lowered (P<0.05) the glucose level from 8.5 ± 1.3 mmol/l to 6.1 ± 1.0 mmol/l at 1 h after the last taurine injection. This decrease was preserved for the next two hours, being 4.3 ± 2.0 mmol/l before the death of the animals. The high SD (2.0 mmol/l) at this last time-point results from substantial differences in blood glucose levels in mice treated with ethanol and taurine. The individual differences varied between 2.2 and 6.0 mmol/l. Taurine alone lowered blood glucose in the old mice from 8.8 ± 1.0 mmol/l to 5.6 ± 1.8 mmol/l in 1 h after the last taurine treatment. The blood glucose level increased slightly during the next two hours to reach almost to the level before experiments. Ethanol alone did not affect the blood glucose level.
10. Discussion

Apoptosis, or programmed cell death, is essential for normal development and maintenance of health in multicellular organisms. A failure in the regulation of this process impairs the delicate balance between cell proliferation, cell survival and cell death. Normally apoptosis eliminates dangerous, unwanted and mutated cells. When the cell death rate is impaired the condition will lead to such diseases as tumor growth and cancer. On the other hand, excessive uncontrolled apoptosis causes progressive neuronal loss leading to neurodegenerative conditions such as Parkinson’s or Alzheimer’s diseases. Apoptosis is a complex, tightly regulated process which occurs stepwise via the consecutive activation of specific proteins which destroy the cell (Hotchkiss et al., 2009). This sequence offers an opportunity to interfere with the apoptotic process by means of drugs which intensify it, for example, kill cancer cells or prevent apoptosis and save a number of neurons and delay neurodegenerative processes in the brain. The amino acid taurine is a good candidate means of preventing undesirable apoptosis, 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), is involved in apoptosis regulation (Takatani et al., 2004; Wu J.Y. et al., 2009) and protects many cell types under pathological conditions such as ischemia (Takatani et al., 2004), high glucose level (Ulrich-Merzenich et al., 2007) and oxidative stress (Das et al., 2009). Here we studied the neuroprotective actions of taurine in two different experimental models – ischemia in vitro (I) and alcohol intoxication in vivo (II-IV). We chose to use ischemia-induced apoptosis in vitro to investigate the possible protective effect of taurine for several reasons. First, it has already been shown that taurine protects cardiomyocytes from ischemia-induced apoptosis (Takahashi et al., 2003), and we decided to test the possible protective effects of taurine in brain cells. Ischemia induces taurine release from neurons and it has been suggested that it could be an important mechanism against excitotoxicity, protecting the brain under cell-damaging conditions (Saransaari and Oja, 2000, 2007).
10.1 Methodological Aspects

We selected the SON and PVN of the hypothalamus as study objects, because they have high levels of taurine (Palkovits et al., 1986), which may underlie the resistance of neurons to damage in these structures. The functions of these neurons are regulated by taurine, which exerts an inhibitory effect on their activity (Hussy et al., 1997, 2000), i.e., they are sensitive to taurine administration. The protective effects of taurine may be mediated by membrane receptors and this was another reason to use SON and PVN neurons. In contrast to experiments in vivo, experiments in vitro allowed us to study directly the effect of taurine on a selected brain area and provided better control of the taurine concentration, which remained constant throughout the experiments. Unfortunately, our experimental model of ischemia in vitro has its own limitations. We showed the effects of taurine on the expression of initiator caspases – caspase-8 and caspase-9 – which were assayed 3 h after ischemic conditions. However, to test whether taurine prevents DNA fragmentation (a final step of cell death) it would be necessary to continue incubation for 12-24 h (Cao et al., 2001), but this was impossible with our in vitro technique. Nor did the in vitro model reflect correctly the real situation when drugs are given through systemic routes in vivo (orally or via the blood). To investigate the possible protective effects of taurine in late apoptosis we used the in vivo model of acute ethanol intoxication, which has been developed and well described by other authors (Ikonomidou et al., 2000; Dikranian et al., 2005). We adopted this model for our study of taurine neuroprotection (II-IV). 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 time frame of which varies in different species, being the last trimester of pregnancy in humans and the first postnatal week in mice (Rice and Barone 2000). In our experiments we used mice during the first postnatal week as a rough model of human FAS (Ikonomidou et al., 2000, 2001). As an object of study we chose the cerebellum, whose simple layered structure made it attractive for histological examination. Moreover, it contains different types of cells, including inhibitory Purkinje cells and excitatory granular cells (Fonnun and Lock, 2000). Taking into account that taurine is an inhibitory amino acid (Oja et al., 1977) we could expect to find different effects of taurine on Purkinje and granular neurons.
By reason of the difference in the time frames of vulnerability for Purkinje cells and granular cells (Dikranian et al., 2005), we used mice at P4 to study Purkinje cells and at P7 to study granular cells (from the IGL and EGL of cerebellum) to ensure a maximal toxic effect on these neurons after ethanol administration. We evaluated the rate of apoptosis in the mouse cerebella by counting TUNEL-positive cells in histological slices. The TUNEL assay is considered a good and fairly precise method for the evaluation of apoptosis (Hewitson et al., 2006; Doonan and Cotter, 2013) and is widely used for the detection of cells with DNA fragmentation. However, it should be taken into consideration that TUNEL staining is not 100% specific to apoptotic cells but also detects cells with DNA damage of non-apoptotic origin such as necrotic degenerating cells, cells undergoing DNA repair, cells damaged by mechanical forces and even cells undergoing active gene transcription (Loo, 2011). For more accurate apoptosis detection it is recommended to combine TUNEL staining with other methods (Baima and Sticherling, 2002; Krysko et al., 2008). We therefore took the activated caspase-3 as an additional marker of apoptosis (Huerta et al., 2007). Activation of caspase-3 cannot in fact be considered as a sufficient marker of apoptosis, as it has been shown also to evince non-apoptotic functions of caspases (Miura, 2013). However, the combination of these two methods of apoptosis detection (TUNEL assay and immunohistochemical detection of activated caspase-3) yields more accurate results in apoptosis assays. Moreover, for our goal of evaluating possible antiapoptotic effects of taurine we used a well-proven experimental model of ethanol-induced apoptosis developed by authors who detected apoptosis by several methods including immunohistochemistry for activated caspase-3, TUNEL staining and electron microscopy (Ikonomidou et al., 2000; Dikranian et al., 2005). We can be relatively sure that we evaluated taurine effects on apoptosis rather than other types of cell death. We may now pass on to our findings related to taurine neuroprotection and their possible mechanisms.
10.2 Intracellular Mechanisms of the Anti-Apoptotic Effects of Taurine

It would appear that taurine protects cells via both receptors and intracellular mechanisms. Taurine may exert its neuroprotective function by activating GABA\(_A\) receptors, resulting in the neutralization of glutamate-induced depolarization and thereby preventing activation of NMDA receptors, which are both ligand- and voltage-gated (Leon et al., 2009). Coapplication of both antagonists of glycine and GABA\(_A\) receptors has almost completely blocked the neuroprotective effect of taurine, supporting the receptor-mediated hypothesis of taurine protection (Wang et al., 2007; Ricci et al., 2009). On the other hand, the loss of taurine neuroprotection in experiments in vitro in the presence of the taurine transporter inhibitor guanidinoetane sulfonate, indicates that intracellular mechanisms are also involved in this protective action (Froger et al., 2012).

Two main pathways of apoptosis are well known, receptor-mediated and mitochondria-mediated. The initiator caspases, caspase-8 and caspase-9, correspondingly, play crucial roles in the initiation of these two pathways. First, we may consider the effect of taurine on the caspase-8-dependent apoptosis. There are no previous data available on the effects of taurine on the expression or activation of caspase-8. However, in our present experiments taurine in the incubation medium reduced the caspase-8 expression induced by ischemia in neurons of the SON and PVN in the hypothalamus, displaying an anti-apoptotic effect (I). Interestingly, taurine derivatives have demonstrated controversial effects on caspase-8 and apoptosis in experiments on primary cultures of rat hepatocytes. For example, tauroursodeoxycholic acid reduced the caspase-8 activity induced by glycochenodeoxycholic acid to one half (Schoemaker et al., 2004). Taurocholate also evinced a protective effect on the vagotomy-induced cholangiocyte apoptosis, which was associated with a downregulation of caspases-3, -8, and -9 (Marzioni et al., 2003). On the other hand, taurolithocholate 3-sulfate has induced caspase-8 activation and led to apoptosis in primary cultures of rat hepatocytes (Graf et al., 2002, 2003).

The caspase-9-dependent pathways of apoptosis depend mainly on the integrity of mitochondrial membranes. We should thus consider the roles of factors which induce mitochondrial membrane disruption and the possible protective effects
of taurine associated with this process. One possible mechanism for the anti-apoptotic actions of taurine is its ability to reduce intracellular free Ca$^{2+}$ by inhibiting all types of voltage-gated calcium channels and the NMDA receptor-gated calcium channel (Wu H. et al., 2005; Wu J.Y. et al., 2009) and by increasing Ca$^{2+}$ buffering in mitochondria (El Idrissi, 2008). In this manner it prevents the activation of calpain (a calcium-dependent protease) and protects mitochondrial membranes from disruption (Sun and Xu, 2008; Wu J.Y. et al., 2009). We did not measure the Ca$^{2+}$ levels in our experiments on ischemia- or alcohol-induced apoptosis. However, an excessive accumulation of Ca$^{2+}$ in cells during ischemia (Mattson et al., 2000; Fiskum, 2004) and upon alcohol administration (Michaelis, 1990) has already been shown. It thus seems possible that in our experiments with ischemia- (I) and alcohol-induced (II-IV) apoptosis the anti-apoptotic effects of taurine can be explained by a decrease in intracellular Ca$^{2+}$ level.

Taurine could also protect cells by acting as an antioxidant (Messina and Dawson, 2000; Lourenco and Camilo, 2002), scavenging at physiological concentrations many reactive oxygen and nitrogen species (Oliveira et al., 2010) and preserving cells from the apoptosis induced by reactive oxygen species (ROS) (Biasetti and Dawson, 2002; Eppler and Dawson, 2002). Furthermore, taurine may evince its antioxidant properties indirectly via enhancement of superoxide dismutase (SOD) activity (Higuchi et al., 2012). Ethanol administration to neonatal rats may not induce oxidative stress in cerebellar granular neurons (Kane et al., 2008), in which case we cannot explain the protective action of taurine against ethanol-induced apoptosis by its antioxidative properties (II-IV). However, it has been shown that in ischemia the massive generation of ROS damages the mitochondrial membrane (Mattson et al., 2000; Fiskum, 2004; Warner et al., 2004), which leads to the release of apoptotic proteins and activation of apoptosis through the caspase-9-dependent pathway. In our experiments one possible factor making for protection of hypothalamic cells from ischemia-induced apoptosis is thus the antioxidant property of taurine (I).

For cell survival the balance between the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 is particularly important. A decrease in the Bcl-2 level in cells leads to translocation of Bax to the mitochondria, disruption of their membranes and release of cytochrome C from mitochondria to the cytosol (Hagberg et al., 2009). Application of taurine can restore the pool of Bcl-2 and protect cells
against apoptosis (Wu J.Y. et al., 2009). Since ethanol-induced apoptosis is a Bax-dependent process (Young et al., 2003), we suggest that restoration of the Bcl-2 level was one possible mechanism of apoptosis prevention in our experiments (II-IV).

Taurine may also be able to rescue cells from apoptosis after damage to the mitochondrial membranes and after the release of cytochrome C from mitochondria. In ischemic cardiomyocytes taurine suppresses the formation of the Apaf-1/caspase-9 apoptosome and prevents caspase-9 activation, and thereby preserves cells from apoptosis (Takatani et al., 2004). In our experiments (I) taurine reduced the caspase-9 expression induced by ischemia in neurons of SON and PVN, which is in good accord with these results. Furthermore, in considering the different anti-apoptotic actions of taurine we should mention the gene-regulation hypothesis of Park and his associates (Park et al., 2006a, 2006b). According to these authors taurine affects gene expression and down-regulates the genes involved in signal transduction, cell proliferation and apoptosis. They were the first to study the effects of taurine on gene expression, but the discrete underlying mechanisms are still unknown.

It has been shown that both ethanol (Chen et al., 2008; Ke et al., 2011) and ischemia (Duan et al., 2010) can induce ER stress, which can lead to apoptosis and neurodegeneration (Tabas and Ron, 2011). Recent findings indicate that taurine can protect cells from hypoxia/reoxygenation- and glutamate-induced ER stress through suppression of ER stress markers (Pan et al., 2010, 2012). Inhibition of ER stress could be an additional pathway of taurine in cell protection and apoptosis regulation (Chen et al., 2013). We have also considered the possible anti-apoptotic mechanisms of taurine summarized in Fig. 3.
Taurine may also protect against the adverse effects of ethanol by mechanisms which are not directly related to apoptosis. Alcohol alters physical properties of membrane lipids, changing membrane fluidity and affecting physiologically important membrane enzymes, for instance, Na\(^+\)/K\(^+\)-ATPase activity (Harris and Hitzemann, 1981; Swann, 1987; Sadrzadeh et al., 1994; Maturu et al., 2010). The effects of alcohol on biological membranes depend on the duration of exposure. An acute ethanol administration increases membrane fluidity (Rubin and Rottenberg, 1982; Bjorkman and Jessop, 1994; Gutiérrez-Ruiz et al., 1995), whereas chronic alcohol intake reduces the fluidity of membranes (Goldstein and Chin, 1981; Rubin and Rottenberg, 1982; Gossai and Lau-Cam, 2009), which become more tolerant of the disarranging effect of ethanol (Goldstein and Chin, 1981; Rubin and Rottenberg, 1982). Acute ethanol exposure also diminishes the Na\(^+\)/K\(^+\)-ATPase
activity in the cerebral cortex and brain stem (Rao et al., 1985) and in the kidneys of adult rats (Rodrigo et al., 1998).

The experimental data on the chronic effect of alcohol on the Na⁺/K⁺-ATPase activity are largely contradictory. For instance, chronic alcohol intake has enhanced the Na⁺/K⁺-ATPase activity in rat erythrocyte membranes (Sadrzadeh et al., 1994) and in the brain (Guerri et al., 1978). However, in other studies the activity of Na⁺/K⁺-ATPase was significantly decreased in human erythrocyte membranes after chronic alcohol consumption (Maturu et al., 2010) and in the brain and cerebellum of rat offspring upon chronic alcohol exposure in utero (Rudeen and Guerri, 1985). Taurine also acts as a membrane stabilizer and restores the depletion of Na⁺/K⁺-ATPase activity due to ozone exposure and prevents the depletion of enzyme activity due to cholesterol enrichment (Qi et al., 1995). Furthermore, taurine reduces the fluidity of biological membranes when co-administered with calcium (Nakashima et al., 1996). It thus seems possible that taurine, in addition to its anti-apoptotic actions, can also reduce the adverse effects of acute ethanol administration, acting as a membrane stabilizer by reducing membrane fluidity and restoring Na⁺/K⁺-ATPase activity.

However, the above considerations of possible mechanisms of taurine neuroprotection do not explain why taurine protects granular neurons from the EGL and IGL on P7 and do not affect apoptosis of Purkinje cells on P4. One explanation may be the different functional properties of the neurons studied. The granular neurons in the EGL and IGL are glutamatergic while the Purkinje cells are GABAergic (Hashimoto and Hibi, 2012). Ethanol can induce apoptosis in the developing brain by dual mechanisms, by either blocking NMDA receptors and/or by excessively activating GABA A receptors (Ikonomidou et al., 2000, 2001; Olney et al., 2000). Taurine itself is an inhibitory amino acid which mimics GABA actions (Oja and Saransaari, 2007; Saransaari and Oja, 2008). In that case taurine, by activating GABA A receptors (Wu et al., 2008; Song et al., 2012), could even increase apoptosis, acting in the same way as ethanol. However, we observed no increase in ethanol-induced apoptosis after taurine treatment. On the other hand, taurine inhibits the glutamate-induced Ca²⁺ elevation (Chen et al., 2001). Another simple explanation for the various effects of taurine on ethanol-induced apoptosis in Purkinje cells and granular cells from the EGL and IGL may be the difference in time points at which the neuronal populations were studied. We studied P4 mice at
12 hours and P7 mice at 8 hours after the first ethanol administration. Taurine is readily excreted in the urine and its plasma concentration thus decreased during the experiments (III). At 8 hours the concentration was still relatively high but at 12 hours almost at control level; this was not high enough to protect Purkinje cells.

10.3 Combined Toxicity of Taurine and Ethanol and Its Possible Mechanisms

In addition to the protective actions of taurine against ischemia (Sun and Xu, 2008) and alcohol (Park et al., 2009) toxicity, there is growing evidence of other beneficial effects in Huntington's disease (Tadros et al., 2005), ammonia-induced impairment (Chepkova et al., 2006), anxiety (Kong et al., 2006), cadmium toxicity (Sinha et al., 2008), oxidative stress (Parildar-Karpuzoglu et al., 2008; Higuchi et al., 2012) and renal disorders (Han and Chesney, 2012), for example. It is well known that ethanol at high doses can induce death (Lamminpää, 1994; Church and Witting, 1997; Sanap and Chapman, 2003; Jones et al., 2011), but little has been established as to the possible toxicity of taurine or of the combination of taurine with ethanol or other drugs. In dogs, a single intravenous dose of 2 g/kg taurine has proved safe and during a 14-day period of observation no animals died or showed signs of toxicity (Nishizawa et al., 1991). In Wistar rats, taurine at a single dose of 7 g/kg (intravenously) or 5 g/kg (orally) has had no toxic effect on the animals, which also survived for 14 days without any signs of toxicity or abnormality upon subsequent autopsy (Kihara et al., 1991). These studies show that taurine administered alone is minimally toxic, as was also confirmed in our present experiments (V). We have found only one old study in which a single subcutaneous injection of taurine to adult mice at doses of 7.5 g/kg and 6.0 g/kg proved 100 % and 50 % lethal, respectively (Goldberg and Jefferies, 1946). In our experiments (V) taurine alone administered to 7-day-old mice at a dose of 6 g/kg was not toxic to the animals, possibly because injected in two half-doses 4 h apart. Taurine from the first injection was then already partly incorporated into bile salts or secreted into the urine, as reflected in the decrease in taurine levels in the blood (Lallemand and De Witte, 2004; III).
Taurine is a common component of alcoholic and nonalcoholic energy drinks (Lutmer et al., 2009; Ayala et al., 2009; Higgins et al., 2010). Studies on these beverages and human health might afford a good source of data on the combined effects of taurine with alcohol and other drink components. However, the studies available on the possible adverse effects of energy drinks have focused mainly on caffeine overdoses (Reissig et al., 2009; Seifert et al., 2011; Rath, 2012; Wolk et al., 2012, Gunja and Brown, 2012) and on the adverse effects of the combination of caffeine and alcohol (Weldy, 2010; Wolk et al., 2012). They provide no evidence as to the possible toxicity of taurine alone or in combination with alcohol (Rath, 2012). Our present findings (V) are thus the first proof of the combined toxicity of taurine and ethanol, emphasizing the potential danger of energy drinks for humans. We cannot of course directly extrapolate our findings in mice to the combined toxic effect of taurine and alcohol in humans, by reason of the huge doses of taurine and ethanol used in our experiments. They do not reflect the real concentrations of these drugs in humans consuming energy drinks. For example, the minimal concentration of taurine which induced death of 7-day-old mice when combined with ethanol was 4 g/kg. A young person weighing 50 kg has then to consume about 200 g of taurine. The usual content of taurine is 400 mg per 240 ml energy drink can, which would be the equivalent of about 500 cans or 120 liters of taurine energy drinks to reach the same ratio of taurine to body weight. It seems that water intoxication would kill the person rather than taurine (Farrell and Bower, 2003; Hiramatsu et al., 2007). The situation is similar with the ethanol doses used in our experiments. For 7-day-old mice we used 5 g/kg ethanol and 100 % of the mice survived perfectly, whereas for human children a dose of ethanol of 3 g/kg is fatal (Jepsen and Ryan, 2005). However, such limitations are unavoidable in studies on animals by reason of the differences in sensitivity between humans and animals. The difference in sensitivity to ethanol was already mentioned. Another good example is nicotine. A probable lethal oral dose of nicotine in humans is less than 5 mg/kg, while in rats the 50 % lethal dose is 50-60 mg/kg. Humans are thus at least 10 times more sensitive to nicotine toxicity than rats (Karaconji, 2005). The existence of such differences between different species allows us to suggest that the lethal dose of a taurine and ethanol combination could also be significantly lower in humans than in mice.
It would seem appropriate at this point to discuss one recent publication describing ethanol and taurine toxicity in chick embryos (Berning et al., 2013). The authors, relying on their experimental data, affirm that very small doses of taurine alone or combined with ethanol can be very dangerous to humans. They warn of the potential adverse effect of taurine-containing energy drinks and even provide a recalculation whereby if a pregnant woman weighing about 140 pounds (63.5 kg) consumes a single drink of alcohol mixed with an energy drink containing 1000 mg taurine (7990 μmol), she will be receiving an exogenous taurine dose of 125.8 μmol mixed with ethanol. This dose is 31.5-fold higher than 4 μmol exogenous taurine/kg egg used in this study, which caused apoptosis and membrane lipid peroxidation in chick brains at mid-embryogenesis. The authors fail to mention that they have also found taurine to be markedly more toxic than ethanol. Their figures show that taurine alone at a dose of 4 μmol/kg egg (0.5 mg/kg) induced the same caspase-3 activity as ethanol alone at a dose of 3 mmol/kg egg (138 mg/kg). In other words, taurine is 276-fold more toxic than ethanol and even more toxic than potassium cyanide, which has induced apoptosis at a dose of 6 mg/kg given twice a day (Zhang et al., 2010). It would appear that Berning’s group was mistaken in their dose calculations, or possibly the experimental procedure was inappropriate, because their results do not seem reliable.

We began our experiments on the combined toxicity of taurine and ethanol in 7-day-old mice for the reason that we came upon this phenomenon accidentally in mice of this age. When we sought to increase the taurine dosage to rescue more neurons from ethanol-induced apoptosis in these mice, used as a model of FAS, we observed that a further increase in taurine dose led to the death of the animals. Thereupon, in order to test the hypothesis that the lethality of a taurine and ethanol combination pertains not only to developing 7-day-old mice, which at this age are highly sensitive to ethanol toxicity (Ikonomidou et al., 2000) we repeated our experiments on adult and old mice. We established that the maximal doses of ethanol used for adult (8 g/kg) and old (6 g/kg) mice did not induce mortality (V). Our present data (8 g/kg) differ slightly from the results of another research group (Schechter and Meehan, 1995) with regard to the ethanol dose which does not induce death in young adult mice (6 g/kg), probably by reason of differences in ethanol sensitivity between the two strains of mice tested. Our data on differences in the toxicity of different doses of ethanol in adult and old mice are similar to those
obtained on rats, in which the 50 % lethal dose for 3-4-month-old rats was significantly higher than that for 10-12-month-old rats (Wiberg et al., 1970). In adult and old mice the combined toxicity of taurine and ethanol sufficient to induce 100% mortality was significantly higher than in 7-day-old mice. Sensitivity to the combined toxicity of taurine and ethanol is thus age-dependent (V). It could be partly explained by the difference in alcohol dehydrogenase activity in the liver of young and adult animals. It has been shown in rats (Sjöblom et al., 1978) and in humans (Pikkarainen and Räihä, 1967) that the activity of alcohol dehydrogenase is relatively weak during the early neonatal period and then gradually increases to its maximum in adults. The higher sensitivity of old compared to adult mice to the combined toxicity of ethanol-taurine in our experiments may be explained by the reduction in relative liver size and by the ethanol-induced glutathione depletion taking place with increasing age, as has been shown in rats (Kim et al., 2003). The decrease in glutathione induced by ethanol in old animals could therefore lead to a greater vulnerability of the liver to the oxidative stress invoked by various agents, including ethanol itself.

Taurine treatment significantly attenuates the stress-induced elevation in blood glucose in rats (Nakagawa and Kuriyama, 1975). Taurine exerts a hypoglycemic action in glucose supplementation-induced hyperglycemia (Kulalowski and Maturo, 1984; Kaplan et al., 2004). These findings on the ability of taurine to reduce blood glucose and the observation that ethanol can also lower it (Huang and Sjöholm, 2008) allow us to assume that the lethality of taurine and ethanol co-administration can be induced by hypoglycemia. In keeping with such an assumption, the blood glucose level after taurine and ethanol co-administration was significantly decreased in adult and old mice. In adult mice the decrease in glucose concentration was still far from the dangerous level. However, the old mice could be divided into two subgroups, one of which had a blood glucose level of about 6 mmol/l and the other 2.2 mmol/l, which is very low and may be the reason for death. We suggest that this group difference could explain why only 70 % of the taurine- and ethanol-treated mice died. The remaining 30 % may have had a less dangerous level of blood glucose (6 mmol/l). Hypoglycemia may thus be one reason for the lethality of a taurine and ethanol combination (V) and also one of the reasons for its age-dependency. In young children ethanol causes hypoglycemia and hypoglycemic seizures (Ricci and Hoffman, 1982; Lamminpää and Vilska, 1990; Yang et al.,
Ethanol inhibits gluconeogenesis (Field et al., 1963; Madison et al., 1967) and since the glycogen stores in the livers of neonatal rats are markedly smaller than in adults (Vernon and Walker, 1972), acute ethanol administration induced hypoglycemia more easily in young animals than in adults (Hollstedt et al., 1980).

Our observations on dying animals and preliminary experiments show that the mechanisms underlying the toxicity of taurine and alcohol combined are not limited to the drop in blood glucose level. We noted that animals from the different age groups treated with taurine and ethanol displayed signs of a marked decrease in heart beat rate and a suppression of respiration. We therefore suggest that the decrease in heart rate and the depression of respiration could be two further mechanisms involved in the lethality of an ethanol-taurine combination. Such a conception is corroborated by the well-known fact that ethanol at high doses has a depressive effect on the CNS, which manifests itself in lowered heart and breathing rates (Sahn et al., 1975; Lamminpää and Vilska, 1990; Pagala et al., 1995), and ethanol can indeed even induce death by respiratory depression (Church and Witting, 1997; Paton, 2005). Data indicating that taurine lowers the heart rate (Bousquet et al., 1981; Paakkari et al., 1982; Wessberg et al., 1983) and respiration (Paakkari et al., 1982; Holtman et al., 1983; Wessberg et al., 1983) also are in agreement with this hypothesis, which of course requires further experimental confirmation, as planned for the immediate future.
11. Summary

1. In vitro taurine (20 mM) preserves neurons in the supraoptic and paraventricular nuclei of the adult mouse hypothalamus from both extrinsic and intrinsic pathways of ischemia-induced apoptosis by reducing the expression of the key pro-apoptotic signalling proteins caspase-8 and caspase-9.

2. In vivo taurine (2 g/kg) saves about 50% of dying immature neurons in the external and internal granular layers of the cerebellum from ethanol-induced apoptosis. However, the same taurine treatment has no protective effect against ethanol-induced apoptosis in the Purkinje cells.

3. This work is the first report on the novel phenomenon of the combined toxicity of taurine and ethanol. This adverse effect is age-dependent. The lethal effect of taurine and ethanol co-administration can be related to a drop in blood glucose.
12. Conclusions and Implications

Our findings in the field of taurine neuroprotection allow us to draw several conclusions. First of all, we have shown that taurine may have beneficial effects against ischemia-induced brain damage, suppressing both extrinsic and intrinsic apoptotic pathways by reducing the expression of the key pro-apoptotic signalling proteins caspase-8 and caspase-9. We have also shown the protective effect of taurine against alcohol-induced neuronal loss in the developing cerebellum. This finding proposes a potential benefit of taurine treatment, alleviating the consequences in fetal alcohol syndrome. Taurine may exert its neuroprotective action by different extracellular and intracellular mechanisms, including activation of GABA_A receptors, regulation of intracellular Ca^{2+} level, scavenging of free radicals, prevention of apoptosome formation and caspase-9 activation, suppression of endoplasmic reticulum stress signalling pathways, restoration of the balance between pro- and anti-apoptotic proteins, and possibly others. The beneficial properties of taurine, its abundance in the human body and its very low toxicity make it attractive for medical purposes. However, one needs to keep in mind the admonition of Paracelsus – the dose makes the poison. Our findings on the combined toxicity of taurine and ethanol suggest that the use of taurine for human treatment should be undertaken with precautions as yet not charted. Other substances combined with taurine may be more toxic than taurine alone. Our work is the first report on the novel phenomenon of the combined toxicity of taurine and ethanol. The adverse effect is age-dependent. The lethal effect of taurine and ethanol co-administration can be related to a drop in blood glucose, although a decrease in the heart beat rate and the suppression of respiration should be also considered as possible reasons of the death of animals. The present findings may constitute a warning of the interactions of taurine and ethanol and their combined toxic effects, particularly in young people mixing taurine-containing energy drinks with alcohol.
At the end of this thesis book I would like to say "thank you" to all the people who helped me during my work and without whom it would have been impossible to start, to continue and/or to complete this book. During my long (more than 9 years) work in Finland I met many people who were kind to me and helped me considerably with advice and friendly attitude. I am afraid to miss any of them in my acknowledgments, so please, if you helped me and I do not mentioned you, it does not mean that your help was not valuable or I am an ungrateful person. It means that by chance your name went out from my brain during the time of preparing this section of my thesis. And I am deeply sorry for this regrettable omission.

This study was carried out at the Department of Physiology in the Medical School of the University of Tampere. I take the opportunity to thank the Finnish Center for International Mobility (CIMO), the competitive research funding of the Pirkanmaa Hospital District, the Finnish Cultural Foundation, and the Maud Kuistila Memorial Foundation for financial support for my research, and especially I would like to express my gratitude to the Finnish Foundation for Alcohol Studies, which was particularly generous in its support.

Above all, I would like to thank my parents, my mother Mrs. Tamara Taranukhina and my father Mr. Grigory Taranukhin, without whom it would have been impossible to write this thesis. They made the most valuable impact in my work, they granted me my life. It is simple - no life, no thesis. So, to grant me my life would be a sufficient gift, but they went further and worked hard to support me during my studies at school, at the University and even later during my postgraduate studies at the Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences. Unlike many other students I did not need to work to make money for food or other expenses and I could focus entirely on my education. Special thanks go to my mother and my mother-in-law Natalia Khokhlova, who found time to come from Russia to Finland to visit us and
to take care of my family when I went abroad to conferences or was much absent engrossed in my time-consuming experiments. At the same time I would also like to thank my brother Alexey Taranukhin and my brother-in-law Alexey Khokhlov for their friendly support.

I also would like to mention the part played by my grandmother Yanina (Nina) Pavlovna Oganesova. Unfortunately, she passed away a long time ago while I was a second-years student at the Herzen State Pedagogical University in Saint-Petersburg in Russia, so she has no direct involvement in this thesis, but she always supported my strange idea of becoming a scientist during my studies at school and University.

Now, I would like to express my deepest gratitude to my two supervisors – Professor Simo Oja and Professor Pirjo Saransaari – for their delicate supervision of my work. They gave me a great deal of freedom in planning the experiments, helped me to solve the problems I met during my experimental work and helped me with the interpretation and discussion of the results, and with English when preparing manuscripts to be published. They provided me with everything I needed, including large numbers of mice, expensive reagents and devices, and they supported me financially when I was unsuccessful with my own research grant applications. They also gave me many possibilities to attend domestic and international meetings to present our new research findings. To Professor Simo Oja I want to say that I am proud that not many post-graduate students may boast that they have a supervisor who has three doctoral degrees in three different fields, medicine, biochemistry and law. My family and I are also very grateful to Professors Simo Oja and Pirjo Saransaari for the strange looking but very tasty mushrooms named in Finnish “suppilovahvero”, which they kindly and generously supplied us with every autumn.

I would like to thank the former supervisor of my Candidate of Science dissertation Dr. Elena Chernigovskaya for the theoretical and practical knowledge of histological techniques and immunohistochemistry of which I made ample use in my present work.

I am grateful to Professor Markku Pelto-Huikko for his assistance and advice in immunohistochemistry and light microscopy.

I thank my colleague Dr. Svetlana Molchanova, who was the first person from whom I learned the mysterious word taurine and who told me about Professor Simo Oja and Professor Pirjo Saransaari and their laboratory in Tampere. To be
honest I had also at that time never heard of the place called Tampere. Svetlana also told me about the Finnish-Russian CIMO Winter School, which later became a springboard for me to my new life in Finland.

I thank my colleague Dr. Irina Podkletnova for her invaluable assistance in my experiments. I would like to include here Docent Vince Varga, who sadly passed away almost three years ago, for his friendly attitude to me and his valuable advice.

I am deeply grateful to my friends and colleagues from the Medical School – Professor Allan Kalueff, Dr. Anna Minasyan, Dr. Nadya Nazarova, Dr. Anastasia Galeeva and Dr. Sergey Stroev, who helped me in many ways to adapt to the life and work in Finland.

Especially I would like to thank my friend and colleague Professor Allan Kalueff for his kindness, keen humour and interesting discussions which we had had over a cup of coffee in the evenings at the Medical School. As a post-graduate student Allan worked hard and quite often spent his nights in the animal house doing his time-consuming behavioural tests. He completed his PhD thesis during two years, then went to the United States and is now professor in his own laboratory. He was and is for me a good example of how to work as a real scientist. I always admired his hard-working character, understanding at the same time that I myself will never work that hard for the simple reason that I prefer to spend my nights at home.

I would also like to thank my friends and colleagues from Russia, Dr. Evgeny Olshevsky and his wife Dr. Natalia Berezneva, as well as Dr. Sergey Nesterov and his wife Dr. Lubov Yamova, for their friendship and kindly support.

I thank Dr. Tiina Solakivi, who offered invaluable help with blood glucose determinations.

I am deeply grateful to Mrs. Raija Repo, Mrs. Irma Rantamaa, Mrs. Oili Pääkkönen and Mrs. Ulla M. Jukarainen for excellent technical assistance. I thank Mrs. Sari Luokkala for solving all the problems I have had with my computer.

My special thanks go to all workers in the animal house for their generous help.

I would like to thank my English teachers Michael Belin, Mike Horwood, Kate Moore and Alisdair Mclean, who encouraged me in my efforts to speak English, to discuss in English and to write presentations in English.
I express my sincere gratitude to my reviewers Dr. Irma Holopainen and Professor Hannu Alho for their careful revision of my thesis and their valuable comments and criticism, which helped me significantly to improve its quality. I also sincerely thank Mr. Robert McGilleon for his revision of the English language in this thesis.

Last in the text, but not last in the meaning, I would like to thank my small family. First of all, my wife Elena Taranukhina for her love and unwavering confidence in me. She takes care of everything in our home – shopping, cooking, washing, cleaning, taking care of me and the children, so doing everything a good housewife can do. Also she helped a lot (and I hope she will help me in the future) in preparing microscope pictures and graphs for publications, graphs and pictures for presentations and in many other ways, including organizing my own work, being at the same time co-author, colleague, secretary, friend and wife. And a word for my sweet children – my 6-years-old son Ivan and my 2-years-old daughter Margarita Kirsi Fiona - who make my life very exciting. They are always funny, noisy, naughty as normal children should be, and it does not matter how tired I sometimes am, they always force me to smile and I am proud to be their father. Now I would like to say some words about the new member in my family, a Norwegian forest cat named Keke. I used him as a stress-relief during the last hard month while I completed the thesis and I shall use him in the same way in future. I hope this is not in contravention of the Finnish law on animal use, but he never complains, although I have no Norwegian to understand him.

In conclusion, I would like to thank Paracelsus, who invented that phrase about 5 hundred years ago, which in the best possible way suits as an epigraph to my thesis. This is amazing!

Tampere, October 2013

Andrey G. Taranukhin
14. References


Kontro P, Korpi ER, Oja SS (1990) Taurine interacts with GABA \textsubscript{A} and GABA \textsubscript{B} receptors in the brain. Prog Clin Biol Res 351:83-94.


Lavrik IN, Krammer PH (2012) Regulation of CD95/Fas signaling at the DISC. Cell Death Differ 19:36-41.


immunoreactivity in the cerebellum of the rat with monoclonal antibodies against taurine. J Neurosci 8:4551-4564.


96


Wu C, Miyagawa C, Kennedy DO, Yano Y, Otani S, Matsui-Yuasa I (1997) Involvement of polyamines in the protection of taurine against the cytotoxicity of

15. Original Communications
Taurine reduces caspase-8 and caspase-9 expression induced by ischemia in the mouse hypothalamic nuclei

Andrey G. Taranukhin¹²*, Elena Y. Taranukhina³, Pirjo Saransaari¹, Irina M. Djatchkova¹, Markku Pelto-Huikko⁴, and Simo S. Oja⁵

¹ Brain Research Center, University of Tampere Medical School, Tampere, Finland
² Laboratory of Comparative Somnology and Neuroendocrinology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St.Petersburg, Russia
³ School of Public Health, University of Tampere, Tampere, Finland
⁴ Department of Developmental Biology, University of Tampere Medical School and Department of Pathology, Tampere University Hospital, Tampere, Finland
⁵ The Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland
Summary. Taurine is a sulphur-containing amino acid abundant in the nervous system. It protects cells from ischemia-induced apoptosis, but the mechanism underlying this is not well established. The aim of our study was to explore the effects of taurine on two main pathways of apoptosis induced by ischemia: receptor-mediated and mitochondrial cell death. Brain slices containing the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus were incubated in vitro under control and simulated ischemic (oxygen-glucose deprivation for 30 min) conditions in the absence and presence of 20 mM taurine. Brain slices were harvested after the 180-min “postischemic” period and fixed in 4 % paraformaldehyde. To estimate apoptosis, immunostaining was done for caspases-8 and caspase-9 in paraffin-embedded sections. Immunoreactive caspase-8 and caspase-9 cells were observed in SON and PVN in all experimental groups, but in the “ischemic” group the expression of caspase-8 and caspase-9 and the number of immunoreactive cells was significantly increased in both hypothalamic nuclei. Addition of taurine (20 mM) to the incubation medium induced a marked decrease in caspase-8 and caspase-9 immunoreactivity after ischemia in SON and PVN when compared with the taurine-untreated “ischemic” group. Taurine reduces ischemia-induced caspase-8 and caspase-9 expression, the key inducers of apoptosis in SON and PVN.

Keywords: Taurine – ischemia - caspase-8 -caspase-9 – hypothalamus - brain slices - mice

Abbreviations: aCSF, artificial cerebral spinal fluid; DAB, diaminobenzidine; GL, grey level; IR, immunoreactive; PBS, sodium phosphate buffer; PVN, paraventricular nucleus; SON, supraoptic nucleus
Introduction

Ischemic injury causes severe neurodegeneration and consequently a loss of normal brain functions (Schwartz et al., 1998; Block, 1999; Lipton, 1999). The mechanisms underlying neuronal degeneration and the potential neuroprotective effects of some pharmacological treatments have been actively studied. In different animal models cells dying after ischemic injury exhibit both necrotic and apoptotic features (Lipton, 1999; Liou et al., 2003). In the ischemic core, cell death is due to necrosis (Garcia et al., 1995) and it is difficult, even impossible, to preserve the necrotic cells. In the penumbra, however, dying cells display morphological manifestations of apoptosis (Linnik et al., 1993; MacManus et al., 1993) and with the right treatment they may be saved (Goto et al., 1990; Linnik et al., 1995). Caspases are a family of aspartate-specific cysteine proteases involved in various pathways of programmed cell death and play a crucial role as initiators or effectors of apoptosis (Earnshaw et al., 1999; Budihardjo et al., 1999). Evidence is mounting to indicate a major role of caspases in ischemia-mediated cell death (Thornberry and Lazebnik, 1998; Love, 2003). Caspases begin to be expressed at high levels and become activated under post-ischemic conditions. For this reason, genetic knockout or pharmacological block of caspases seems to be effective in reducing cell damage in both in vivo and in vitro models of ischemia (Le et al., 2002).

There are two main pathways of caspase activation: death receptor-mediated and mitochondrial. Death receptor-mediated apoptosis is initiated by activation of caspase-8 from cell surface receptors. Caspase-8 subsequently causes direct activation of caspase-3 (Scaffidi et al., 1998) or/and activates downstream caspases, which destroy cells (Budihardjo et al., 1999). The mitochondrial pathway of programmed cell death involves the release of cytochrome C, procaspase-9, and Apaf-1 from the mitochondrial intermembrane space and a series of subsequent biochemical interactions, including the activation of caspase-9 and thereafter
activation of caspase-3 (Earnshaw et al., 1999; Budiardjo et al., 1999). Both apoptotic pathways are involved in brain ischemia (Velier et al., 1999; Love, 2003).

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid abundant in the nervous system. It plays an important role in the modulation of neurotransmitter release, calcium homeostasis, osmoregulation and neuroprotection (Oja and Saransaari, 1992; Birdsall, 1998; Saransaari and Oja, 2000). Taurine has been reported to protect the myocardium (Milei et al., 1992) and cardiomyocytes (Takahashi et al., 2003) from ischemia-induced damage, but the mechanism of this protection remains unclear. We now investigated how taurine affects the expression of caspase-8 and caspase-9 in the supraoptic (SON) and paraventricular (PVN) nuclei, chosen as a model of ischemic neurodegeneration because they have high levels of taurine (Palkovits et al., 1986), which may underlie the resistance of these neurons to damage.

Material and methods

Preparation and incubation of slices

Young adult (3-month-old) male NMRI mice were decapitated and their brains rapidly excised and placed in ice-cold oxygenated (95 % O₂, 5 % CO₂) artificial cerebral spinal fluid (ACSF) containing (in mM) 126 NaCl, 5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 2.4 CaCl₂, 10 D-glucose, and 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (Hepes), pH 7.4. Slices containing SON and PVN (400-500 μm thick) were manually prepared and preincubated (two slices in each chamber) in 30 ml ACSF bubbled with 95 % O₂ and 5 % CO₂ in a shaking water bath at 37°C for 30 min. After the preincubation period, the slices were divided into 3 experimental groups. Those in the first (“control”) group were incubated in the above-mentioned conditions until the end of experiments. The slices in the second (“ischemic”) group were
incubated for 30 min in ACSF without glucose and bubbled with N₂ to mimic ischemic conditions. The third group (“ischemia + taurine”) were incubated for 30 min in ACSF with taurine (20 mM) without glucose and bubbled with N₂. We used this high taurine concentration because it has been shown that 20 mM of taurine most effectively protects tissue against apoptosis and cell loss (Eppler and Dawson, 2002; Takahashi et al., 2003; Takatani et al., 2004).

After the 30-min “ischemic” period, the medium was replaced every 30 min with fresh ACSF bubbled with 95 % O₂ and 5 % CO₂ to simulate a “reperfusion” period. In the “ischemia + taurine” group taurine was present during all periods of incubation. Slices were removed at 180 min after the end of the “ischemic” period and fixed in 4 % paraformaldehyde in sodium phosphate buffer (PBS) (0.1 M, pH 7.4) overnight at 4°C.

Conventional histology

Paraformaldehyde-fixed slices were embedded in paraffin and cut with a microtome into 5 μm-thick sections. The sections were mounted on glycerin-albumin-coated slides and dried overnight at 37°C. Hematoxylin/eosin staining was used to demonstrate the general histoarchitecture of the slices after incubation in vitro. Deparaffinized sections were hydrated over the xylene and ethanol series to distilled water and stained with hematoxylin, washed, stained with eosin, washed again and dehydrated using ethanol and xylene for embedding.

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 10 mM citric acid buffer (pH 6.0)], washing in 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 specimens were incubated with polyclonal caspase-8 (Caspase-8 (FLICE) Ab-4, Lab Vision Corp., diluted 1:200 in serum-blocking solution) or polyclonal caspase-9 antibody (sc-8355, Santa Cruz Biotechnology, Inc, diluted 1:200 in serum-blocking solution) in moist chambers overnight at 4°C. After incubation with 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 caspase-8 and caspase-9 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.

Semi-quantitative analysis of caspases

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) program was used for a semi-quantitative analysis of caspase-8 and caspase-9 expression in the histological sections of SON and PVN of the hypothalamus. Five sections cut at the same level of each hypothalamic nucleus from every animal were analyzed. The sections were reviewed at 250-fold magnification under light microscope. Optical density was evaluated by two parameters reflecting the expression level of this protein in the selected hypothalamic nuclei. As the first parameter, the number of caspase-immunoreactive (IR) cells was calculated in every slice and the
average number of caspase-8-IR or caspase-9-IR cells per slice of hypothalamic nucleus 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). Optical density reflecting the content of the proteins studied in neurons was calculated as the GL of an immunoreactive field of cell by subtracting the background GL. The optical density of the background was estimated in the same slice in the field of non-immunoreactive brain tissue.

Statistical analysis

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

Results

Hematoxylin-eosin stained paraffin sections displayed normal parenchymal architecture and cell morphology of SON and PVN. In no experimental group were necrosis and apoptotic bodies morphologically discernible.

Effect of ischemia on the caspase-8 and caspase-9 expression in SON and PVN

Immunoreactive (IR) caspase-8 and caspase-9 cells were observed in SON and PVN in all experimental groups, but in the “ischemic” group their numbers were significantly increased ($p<0.05$) in both hypothalamic nuclei (Fig.1A,B). Moreover, ischemia led to upregulation of caspase-8 expression in individual neurons of both SON and PVN (Fig.2A,B). All these changes indicate that ischemia led to the initiation of two main apoptotic pathways in SON and PVN of
the hypothalamus.

**Effect of taurine on caspase-8 and caspase-9 expression induced by ischemia**

After incubation with taurine, the number of caspase-8-IR cells in PVN was significantly decreased (p<0.05) when compared to PVN cells in the ischemic group (Fig.1B). Taurine also reduced caspase-8 immunoreactivity in neurons of SON and PVN under ischemic conditions (Fig.2A,B). The presence of taurine likewise affected the expression of caspase-9. It reduced the number of caspase-9-IR cells after ischemia in SON and PVN (Fig.1A,B), and the expression of caspase-9 in neurons of SON (Fig. 2A) in comparison with the taurine-untreated “ischemic” group.

**Discussion**

It has been demonstrated that oxygen-glucose deprivation-exposed slices is a useful in vitro model to study many physiological processes (Joshi and Andrew, 2001; Saransaari and Oja, 2002, 2004, 2005) and in particular apoptosis during ischemic brain injury in adult mice (Gottron et al., 1997; Martin-Villalba et al., 2001; Plesnila et al., 2001; Le et al., 2002). Caspase-mediated apoptosis can be induced through two major upstream pathways. The first is via the release of cytocrome C from the mitochondria into the cytoplasm, where it consequently binds to pro-caspase-9 and Apaf-1, forming an apoptosome. This cleaves caspase-9 which further cleaves caspase-3 (Ferrer and Planas, 2003). The second pathway is via the binding of the Fas to the Fas-associated death domain, which activates procaspase-8. Procaspase-8 cleaves caspase-8 which activates caspase-3 (Boatright and Salvesen, 2003). To determine which pathway of apoptosis induction is utilized by ischemia in SON and PVN of the hypothalamus, we measured caspase-8 and caspase-9 expression. The results showed that caspase-8 and caspase-9 expressions
significantly increased in neurons of both hypothalamic nuclei. Our data thus suggest that ischemia induces apoptosis in SON and PVN through both caspase-8 and caspase-9 pathways.

Excessive accumulation of Ca$^{2+}$ occurs during ischemia-mediated mitochondrial injury (Mattson et al., 2000; Fiskum, 2004). Reactive oxygen species generated during ischemia damage the mitochondrial membrane (Mattson et al., 2000; Fiskum, 2004; Warner et al., 2004) lead to release of apoptotic proteins which activate apoptosis through the caspase-9-dependent pathway. Taurine can modulate the intracellular calcium homeostasis by reducing the intracellular calcium level in neurons (Foos and Wu, 2002) and in this manner promote its neuroprotective action. Furthermore, taurine acts as an antioxidant and preserves cells from apoptosis induced by reactive oxygen species (Biasetti and Dawson, 2002; Eppler and Dawson, 2002).

In the first demonstration of the molecular mechanisms of the antiapoptotic effects of taurine it suppressed the formation of the Apaf-1/caspase-9 apoptosome in cardiomyocytes and thereby prevented caspase-9 activation and apoptosis (Takatani et al., 2004). In our experiments taurine reduced caspase-9 expression in neurons of SON and PVN, which is in good accord with the results of Takatani et al. (2004).

There is no previous data on the effects of taurine or taurine derivatives on caspase-8 and caspase-8-dependent apoptosis in neurons. Experiments carried out on a primary culture of rat hepatocytes demonstrated that taurine derivatives exert controversial effects on caspase-8 and apoptosis. For example, tauroursodeoxycholic acid reduced the caspase-8 activity induced by glycochenodeoxycholic acid to one half (Schoemaker et al., 2004). Taurocholate also evinced a protective effect on vagotomy-induced cholangiocyte apoptosis, which was associated with the downregulation of caspase-3, 8, and 9 (Marzioni et al., 2003). On the other hand, taurolithocholate 3-sulfate has induced caspase-8 activation and led to apoptosis in a primary culture of rat hepatocytes (Graf et al., 2002, 2003). In our experiments, taurine in the incubation
medium reduced caspase-8 expression induced by ischemia in neurons of SON and PVN in the hypothalamus. Taurine can thus protect these hypothalamic neurons from caspase-8-dependent apoptosis.

We have now shown that taurine can reduce the expression of caspase-8 and caspase-9 induced by ischemia. What are the possible mechanisms? Caspase-8 resides in the cytosol as an inactive 53-kDa precursor and like all other caspases is activated by proteolytic processes at what constitute caspase consensus cleavage sites. When activated, procaspase-8 zymogen is cleaved into large (~43-kDa) and small (~12-kDa) polypeptides, which assemble to form the heterotetrameric structure shared by all known active caspases (Earnshaw et al., 1999; Kruidering and Evan, 2000). In our work we used the caspase-8 antibody, which detects the inactive 53-kDa precursor and the processed forms (43-kDa, 12-kDa) of caspase-8. The increasing expression of caspase-8 induced by ischemia might thus indicate an increased level of precursor procaspase-8 expression as well as caspase-8 activation. Hence, the reduction of caspase-8 expression by taurine would indicate that taurine can influence the gene expression of caspase-8 or can suppress its activation. The data presented by Park and his associates (Park et al., 2006a, 2006b) corroborate the gene-regulation hypothesis whereby taurine affects gene expression and down-regulates the genes implicated in signal transduction, cell proliferation and apoptosis. Theirs are the first studies on the effects of taurine on gene expression but the detailed mechanisms of these effects are still unknown.

Recently, Matsumori and colleagues (2006) have shown that under ischemic conditions overexpression of heat shock protein 70 increases the expression of cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (FLIP) and decreases caspase-8 cleavage. Taurine enhances the expression of heat shock protein 70 (Kurz et al., 1998) and in
such a manner may prevent the activation of caspase-8, though this assumption is in need of confirmation.

Caspase-9 is synthesized as a 50-kDa precursor protein. Like caspase-8 and other caspases, it consists of three domains: an N-terminal pro-domain, a large subunit (~37-kDa) and a small subunit (~12-kDa). Procaspase-9 is converted to the active form of caspase-9 by cleavage at specific sites between the different subunits. The active caspase-9 enzyme is a heterotetramer containing two small and two large subunits (Earnshaw et al., 1999). The caspase-9 antibody used in our experiments reacts with the large subunit (~37-kDa) and the precursor of caspase-9. We cannot therefore recognize what kind of caspase-9, active form or zymogen, was detected. In any case, the increased caspase-9 level in the cells indicates that its synthesis is enhanced. Consequently, the reduction of the caspase-9 expression in ischemic neurons by taurine suggests that it affects caspase-9 synthesis via down-regulation of the corresponding genes (Park et al., 2006a, 2006b).

Only relatively high concentrations of taurine have been found to be effective when other parameters have been studied with various brain preparations (del Olmo et al., 2000b, Messina and Dawson, 2000, El Idrissi and Trenkner, 2003). The extracellular concentrations of taurine in the brain in vivo are many times higher those of, for instance, amino acid neurotransmitters (Lerma et al., 1986, Miele et al., 1996, Molchanova et al., 2004a). Ischemia itself evokes an approximately 30-fold increase in extracellular taurine (Molchanova et al., 2004b). Furthermore, there is unambiguous evidence that 20 mM of taurine is able to protect most effectively against apoptosis and cell loss (Eppler and Dawson, 2002; Takahashi et al., 2003; Takatani et al., 2004). In keeping with the investigations in question, a rather high concentration of taurine had now to be used to elicit significant reductions in caspase expression.
In conclusion, our findings show that taurine can suppress ischemia-induced apoptosis in hypothalamic neurons of SON and PVN by reducing caspase-8 and caspase-9 expression. Taurine participates in the regulation of the death receptor-mediated and mitochondrial pathways of apoptosis, but the exact mechanisms of its effects on caspase-mediated apoptosis are still unclear and subjected to further studies.

**Acknowledgements**

This study was supported by the Finnish Center for International Mobility (grants EH-04-2542 and MK-05-3549) and the competitive research funding of the Pirkanmaa Hospital District (grants 9F051 and 9F224).

**References**


Authors’ address: Dr. Andrey Taranukhin, Tampere Brain Research Center, Medical School, FI-33014 University of Tampere, Finland,

Fax: +358 3 3551 6170, E-mail: Andrey.Taranukhin@uta.fi
Figure legends

**Fig. 1.** Effect of taurine on the number of caspase-8- and caspase-9-immunoreactive cells in the supraoptic (SON) (A) and paraventricular (PVN) (B) nuclei after ischemia. Open bars show caspase-8 and filled bars caspase-9. Data are mean values ± SEM. * p<0.05 compared with the control group, # p<0.05 compared with the “ischemia” group.

**Fig. 2.** Effect of taurine on the expression of caspase-8 and caspase-9 in neurons of the supraoptic (SON) (A) and paraventricular (PVN) (B) nuclei after ischemia. Open bars indicate caspase-8 and filled bars caspase-9. Data are mean values ± SEM (bars). * p<0.05 compared with the control group, # p<0.05 compared with the “ischemia” group.
Fig. 1.
Fig. 2.
TAURINE PROTECTS IMMATURE CEREBELLAR GRANULAR NEURONS AGAINST ACUTE ALCOHOL ADMINISTRATION

Andrey G. Taranukhin, Elena Y. Taranukhina, Irina M. Djatchkova, Pirjo Saransaari, Markku Pelto-Huikko and Simo S. Oja

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; Spadoni 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, 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 and Bobyock, 1988). Furthermore, ethanol administration has been shown to elicit an increase of extracellular taurine in the rat cerebral cortex and hippocampus (Dahchour 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 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).

1 Brain Research Center, University of Tampere Medical School, Tampere, Finland
2 Laboratory of Comparative Somnology and Neuroendocrinology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St.-Petersburg, Russia
3 Department of Developmental Biology, University of Tampere Medical School, and Department of Pathology, Tampere University Hospital, Tampere, Finland
4 The Centre for Laboratory Medicine and Department of Clinical Physiology and Nuclear Medicine, Tampere University Hospital, Tampere, Finland
2. MATERIALS AND METHODS

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.

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.

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.

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$_2$O$_2$ 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.

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.

3. RESULTS

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 age (Fig. 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. 2).
Figure 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.

Figure 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.
3.2. Effects of Taurine on Ethanol-Induced Apoptosis

TUNEL-positive cells were counted in each lobule of the cerebellar vermis (Fig. 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.

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, 2002b), 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 witnesses 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 in 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 precedes 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 in 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.

5. ACKNOWLEDGMENTS

The authors are deeply grateful to Mrs. Ulla M. Jukarainen for excellent technical assistance. This study was supported by the Finnish Center for International Mobility (grants EH-04-2615 and MK-05-3550) and the competitive research funding of the Pirkanmaa Hospital District (grants 9F070, 9G051, 9G203).

6. REFERENCES


Neuroprotection by taurine in ethanol-induced apoptosis in the developing cerebellum

Andrey G Taranukhin1,2*, Elena Y Taranukhina1, Pirjo Saransaari1, Irina M Podkletnova1, Markku Pelto-Huikko3, Simo S Oja1,4†

From 17th International Meeting of Taurine
Fort Lauderdale, FL, USA. 14-19 December 2009

Abstract

Background: Acute ethanol administration leads to massive apoptotic neurodegeneration in the developing central nervous system. We studied whether taurine is neuroprotective in ethanol-induced apoptosis in the mouse cerebellum during the postnatal period.

Methods: 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 1 h and 2.5 g/kg at 3 h) to the ethanol and ethanol+taurine groups. The ethanol+taurine group also received two injections of taurine (1 g/kg each, at time zero and at 4 h). To estimate 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 12 or 8 hours after the first taurine injection. Changes in the blood taurine level were monitored at each hour by reverse-phase high-performance liquid chromatography (HPLC).

Results: Ethanol administration induced apoptosis of Purkinje cells on P4 in all cerebellar lobules, most extensively in lobules IX and X, and on P7 increased the number of activated caspase-3-immunoreactive and TUNEL-positive cells in the internal layer of the cerebellum. Administration of taurine significantly decreased the number of activated caspase-3-immunoreactive and TUNEL-positive cells in the internal layer of the cerebellum on P7, but had no effect on Purkinje cells in P4 mice. The high initial taurine concentration in blood of the ethanol+taurine group diminished dramatically during the experiment, not being different at 13 h from that in the controls.

Conclusions: We conclude that the neuroprotective action of taurine is not straightforward and seems to be different in different types of neurons and/or requires prolonged maintenance of the high taurine concentration in blood plasma.

Background

A moderate alcohol intake may not be harmful and has even beneficial effects in prevention of cardiovascular diseases, for example [1]. On the other hand, heavy alcohol consumption is associated with the reduced brain mass, neuronal loss, neuropathological changes and results in the impairment of cognitive functions, amnesia, dementia and even a significant increase in mortality [2-4]. In adult rats a short-term increase in the blood ethanol concentration up to 6 g/l has not been toxic to the central nervous system [5]. However, in the developing nervous system the situation is quite different. The blood ethanol concentration above 0.5 g/l in mice during their early postnatal life induces mild apoptotic neurodegeneration [6], which becomes dose-dependently more severe from the concentration of 2 g/l upward [7]. Intrauterine exposure of the human fetus to ethanol due heavy drinking or repeated binge drinking of pregnant women causes a wide spectrum of developmental disorders known as the fetal alcohol syndrome [8,9]. The human fetal brain is particularly

* Correspondence: andrey.taranukhin@uta.fi
† Contributed equally
1 Brain Research Center, University of Tampere Medical School, Tampere, Finland
Full list of author information is available at the end of the article

© 2010 Taranukhin et al; licensee BioMed Central Ltd. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
sensitive to the adverse effects of alcohol during the last trimester of pregnancy, the period of synaptogenesis, also known as the brain growth spurt period. In rodents, the same period of increased sensitivity to ethanol is during the early postnatal period [10]. It has been well documented that acute ethanol exposure to neonatal mice induces neuronal loss by apoptosis [6,7,11-14]. Prevention of ethanol-induced apoptosis can save a huge amount of neurons and significantly decrease the harmful consequences of alcohol intoxication.

Taurine is a simple sulphur-containing free amino acid abounding in electrically excitable tissues such as brain, retina, heart and skeletal muscles [15]. It is involved in a wide range of physiological processes, for example, in osmoregulation, lipid metabolism, intracellular calcium regulation, neuronal development, neuro-modulation and cell protection [16-20]. Recent findings also imply taurine in apoptosis regulation [21-24]. In the present work we focused on the possible protection of Purkinje cells and neurons in the internal layer of the developing cerebellum against apoptosis induced by acute ethanol administration. Among possible means to prevent pathological apoptosis taurine is very attractive since it is a naturally-occurring and non-toxic compound.

Methods

Animals and treatments

Adult NMRI mice for breeding were purchased from Harlan, Netherlands. Four (P4) - and seven (P7) - 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 reduce their number and suffering. The mice in each litter were divided into three groups: ethanol-treated, ethanol+taurine-treated and controls. To induce acute alcohol intoxication 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 1 h and 2.5 g/kg again at 3 h) to the ethanol and ethanol+taurine groups. This dosing regimen was well documented to produce an elevation in the blood alcohol concentration above 2 g/l for at least 8 hours and led to significant and widespread apoptotic neurodegeneration in the developing brain [7] and cerebellum [25]. 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 one hour after the second ethanol injection. The control animals were given saline subcutaneously. Twelve (P4) and eight hours (P7) after the first ethanol injection the mice were killed by decapitation. Blood samples from each animal were collected separately in lithium-heparin tubes and centrifuged at 1750 rpm for 10 min to obtain plasma. The samples were frozen until HPLC analyses. The cerebella were rapidly excised and fixed in 4 % paraformaldehyde in phosphate buffered saline for at least 3 days at 4°C. They were then embedded in paraffin and cut with a microtome into 5-μm thick mid-sagittal sections containing lobules I-X of the cerebellar vermis.

High Performance Liquid Chromatography (HPLC)

The concentration of taurine in the blood serum was measured using HPLC with fluorescent detection after precolumn derivatization with o-phthalaldehyde (OPA) using the analysis equipment system of Shimadzu Scientific Instruments (Kyoto, Japan). The separation column was 4.6 x 250 mm Ulptropac 8 Resin, lithium form (Farmacia, Denmark). Derivatization of taurine was performed with the OPA reagent (0.2 % OPA, 0.1 % mercaptoethanol and 1 % ethanol in 1 M borate buffer, pH 10.4). The elution was done with lithium citrate buffers in the following order: (1) 0.2 M, pH 2.80, (2) 0.3 M, pH 3.00, (3) 0.5 M, pH 3.15, (4) 0.9 M, pH 3.50, and (5) 1.6 M, pH 3.30. Fluorescence of taurine derivatives was measured with an RF-10A detector using the excitation and emission wavelengths set at 340 nm and 450 nm, respectively. The concentrations of taurine were finally estimated using a commercial amino acid mixture (Pickering, UK) as an external standard and diaminonbutyrate as an internal standard.

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 and blocking with 0.5 % hydrogen peroxide in this buffer 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 the above buffer). The specimens were thereafter 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 was used as a chromogen to visualize the sites expressing activated caspase-3 immunoreactivity. The sections for negative control were incubated without the primary antibodies to rule out nonspecific staining. Finally, the sections were counterstained by...
hematoxylin-eosin to better reveal histological details, dehydrated and mounted.

Detection of cell death in situ
DNA fragmentation is one of the most precise markers by which apoptotic cells are recognized. In order to detect DNA fragmentation of cell nuclei, terminal deoxyxynucleotidyl 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$_2$O$_2$ weakens terminal deoxynucleotidyl transferase activity [26] and induces DNA breaks [27]. 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.

Image analysis and cell counting
The sections were processed in parallel under standardized conditions for immunostaining or TUNEL to minimize variability in labeling conditions. 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) was used for analysis of caspase-3 immunoreactivity and TUNEL-staining in the histological sections of the cerebellum. At least five sections cut at the same level of the cerebellar vermis from every animal were analyzed. The amount of cells labeled for active caspase-3 or TUNEL were calculated in every slice in the same level of the cerebellar vermis from every animal. The sections were stained with hematoxylin-eosin to better reveal histological details, dehydrated and mounted.

Statistical analysis
One-way analysis of variance (ANOVA) was used to compare the number of activated caspase-3-immunoreactive 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 < 0.05.

Results
In the present work we focused on the Purkinje cells and neurons in the internal granular cell layer of the cerebellum. The Purkinje cells were identified by their size, shape and specific localization in the cerebellar lobules. Because we did not make specific labeling to identify different types of neurons and glial cells in the internal granular cell layer of the cerebellum, we refer to these cells as internal granular layer (IGL) cells. We studied the possible protective effect of taurine against ethanol-induced apoptosis in the Purkinje cells on P4 and in the IGL cells on P7 mice because of the extremely high sensitivity of these cell types at these ages to ethanol-induced apoptotic neurodegeneration.

Effects of taurine on ethanol-induced caspase-3 activation
Activated caspase-3-immunoreactive (IR) cells in the IGL found in each cerebellar lobule in the saline-treated control mice on P7 evidences physiological cell death which normally occurs at this period of development (Figure 1A, 1D; Figure 2A, 2D; Figure 3A, 3D). Only a few activated caspase-3-IR Purkinje cells were found in the saline-treated control mice on P4.

In the ethanol-treated pups on P4 a large number of activated caspase-3-IR Purkinje cells were discernible in all lobules with the highest amount in lobules IX and X (Figure 4B, 4E). In lobules I-II, III, IV-V, VI-VII and VIII the number of caspase-3-IR Purkinje cells was also significantly increased when compared to the control group (Figure 5A). On P7 ethanol administration induced very little response to caspase-3 activation in the Purkinje cell layer which hardly differed from the control group. However, ethanol treatment on P7 induced widespread activation of caspase-3 in the IGL (Figure 1B, 1E; Figure 2B, 2E; Figure 3B, 3E). The amount of activated caspase-3-IR IGL cells in each lobule was markedly greater than in the saline-treated pups (Figure 5C). The highest immunoreactivity for activated caspase-3 was found in I-II, III and IV-V vermal lobules.

In the ethanol + taurine-treated group on P4 the number of activated caspase-3-IR Purkinje cells in each lobule was approximately the same as in the pups treated only with ethanol and significantly higher than in the saline-treated control group (Figure 4; Figure 5A). However, in contrast to the Purkinje cells at age P4, taurine treatment significantly decreased the number of activated caspase-3-IR cells in the IGL on P7 in each lobule (Figure 1C, 1F; Figure 2C, 2F; Figure 3C, 3F; Figure 5C). The amount of rescued cells from caspase-3 activation were different in different lobules and varied from 34 % to 41 % for lobules VI, VII and X, and from 45 % to 57 % for lobules I-II, III, IV-V, VIII and IX.

Effects of taurine on ethanol-induced DNA fragmentation
A few TUNEL-positive cells in the Purkinje cell layer on P4 and in the IGL on P7 were detected in the saline-treated control groups showing physiological cell death.
Figure 1 Immunostaining for activated caspase-3 in lobule II of the cerebellum of 7-day-old mice 8 hours after the first ethanol injection. A, D: control group, B, E: ethanol-treated group, C, F: ethanol-taurine-treated group. The activated caspase-3-immunoreactive cells in the IGL are indicated by black arrows.

Figure 2 Immunostaining for activated caspase-3 in lobule IV-V of the cerebellum of 7-day-old mice 8 hours after the first ethanol injection. A, D: control group, B, E: ethanol-treated group, C, F: ethanol-taurine-treated group. The activated caspase-3-immunoreactive cells in the IGL are indicated by black arrows.
Figure 3  Immunostaining for activated caspase-3 in lobule X of the cerebellum of 7-day-old mice 8 hours after the first ethanol injection
A, D: control group, B, E: ethanol-treated group, C, F: ethanol+taurine-treated group. The activated caspase-3-immunoreactive cells in the IGL are indicated by black arrows.

Figure 4  Immunostaining for activated caspase-3 in lobule X of the cerebellum of 4-day-old mice 12 hours after the first ethanol injection
A, D: control group, B, E: ethanol-treated group, C, F: ethanol+taurine-treated group. The activated caspase-3-immunoreactive Purkinje cells are indicated by black arrows.
Figure 5 Effects of taurine on ethanol-induced apoptosis in the developing cerebellum (A) Number of activated caspase-3 immunoreactive Purkinje cells in the cerebellar lobules of 4-day-old mice in the control (open bars), ethanol-treated (filled bars) and ethanol+taurine-treated (hatched bars) mice. The results are given per mm² with standard deviations. Number of animals in each group is 5. Ethanol significantly (P<0.01) increased the number of caspase-3 immunoreactive cells in all lobules. There were no significant differences between the ethanol and ethanol+taurine groups. (B) Number of apoptotic Purkinje cells (labeled by TUNEL assay) in the cerebellar lobules of 4-day-old mice in the control (open bars), ethanol-treated (filled bars) and ethanol+taurine-treated (hatched bars) mice. The results are given per mm² with standard deviations. Number of animals in each group is 5. Ethanol significantly (P<0.01) increased the number of TUNEL-positive Purkinje cells in all lobules. There were no significant differences between the ethanol and ethanol+taurine groups. (C) Number of activated caspase-3-immunoreactive cells in the IGL in the cerebellar lobules of 7-day-old mice in the control (open bars), ethanol-treated (filled bars) and ethanol+taurine-treated (hatched bars) mice. The results are given per mm² with standard deviations. Number of animals in each group is 5. Ethanol significantly (P<0.01) increased the number of caspase-3 immunoreactive cells in all lobules. The significance of differences between the ethanol and ethanol+taurine groups: *P<0.05. (D) The number of apoptotic cells (labeled by TUNEL assay) in the IGL in the cerebellar lobules of 7-day-old mice in the control (open bars), ethanol-treated (filled bars) and ethanol+taurine-treated (hatched bars) mice. The results are given per mm² with standard deviation. Number of animals in each group is 5. Ethanol significantly (P<0.01) increased the number of TUNEL-positive cells in all lobules. The significance of differences between the ethanol and ethanol+taurine groups: *P<0.05.
during normal development. The amount of cells undergoing physiological cell death in the Purkinje layer seems to be less than in the IGL and it was in accordance with our data on activated caspase-3 immunoreactivity.

Ethanol treatment significantly increased the number of cells with fragmented DNA labeled by the TUNEL assay in the Purkinje cell layer of P4 mice (Figure 5B). The largest number of TUNEL-positive Purkinje cells was found in lobules IX and X (Figure 6B, 6E), although in the other lobules studied the amount of TUNEL-positive Purkinje cells was also much greater than in the control group. At age P7, ethanol administration induced massive apoptosis in the IGL in all vermician lobules as indicated by the increased amount of TUNEL-positive cells (Figure 5D).

Taurine treatment did not change the amount of TUNEL-positive Purkinje cells on P4 in any lobule studied when compared to the pups treated only with ethanol (Figure 5B; Figure 6). The amount of TUNEL-positive Purkinje cells remained much greater than in the saline-treated control group. Taurine preservation of IGL cells from DNA fragmentation varied in the lobules from 40 % (lobule I-II) to 68 % (lobule IX).

**Dynamics of taurine blood concentration changes during the experiments**

We measured the taurine concentration in blood serum during the experiments from 4 h onwards after the first ethanol injection (1 hour after last taurine injection) at every hour for 12 h and then again at 24 h (Figure 7). In the saline-treated control group and in the ethanol group of P7 mice the taurine concentration remained the same at 4 h, 12 h and 24 h. It was 1.00 ± 0.42 mmol/l in the control group and 0.85 ± 0.24 mmol/l in the ethanol group. Two subcutaneous injections of taurine each at the dose 1 g/kg (one at 0 h and second at 4 h) markedly increased the taurine level up to 13.36 ± 2.73 mmol/l at 4 h after the first ethanol injection. For four hours (4 h-7 h) the concentration in the blood was maintained at about the same high level of 13.20 ± 1.83 mmol/l and then it started to decline gradually and almost reached the control level by 12 h (1.59 ± 0.83 mmol/l).

**Discussion**

The central nervous system is extremely sensitive to alcohol during development and the periods of vulnerability are temporally well defined. The time frames of vulnerability are different for different neuronal populations. In the developing mouse cerebellum acute
alcohol intoxication thus induces massive neuronal death of Purkinje cells on P2-P6 and of cells in the IGL on P7-P9 [25]. In our experiments P4 and P7 mice pups were used to induce ethanol-induced degeneration of Purkinje and IGL cells, respectively. It is well established that alcohol induces neuronal death in the developing brain by apoptosis [11]. Apoptosis is a type of programmed cell death with specific morphological features [28]. Immature neurons dying due to ethanol exposure, exhibit biochemical and ultrastructural features of apoptosis such as activation of caspase-3 [12,13], internucleosomal DNA fragmentation [7,14], clumping of nuclear chromatin, formation of spherical chromatin masses and nuclear membrane fragmentation [7,25,29].

Two major apoptotic pathways have been established: the death-receptor-mediated and the mitochondriamediated apoptosis [28], also known as the “extrinsic” and as “intrinsic” apoptotic pathways, respectively [30]. In both pathways, the caspases, a family of cysteine-dependent aspartate-directed proteases, play an important role in initiation, signal transduction and execution of apoptosis [31,32]. The extrinsic pathway is triggered by activation of death receptors localized at the cell membrane surface and it 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 nitric oxide, oxidants and proapoptotic proteins, e.g. Bax, increase mitochondrial membrane permeability and release cytochrome C into the cytoplasm. Cytochrome C binds to Apaf-1 and procaspase-9, forming an apoptosome, leading to caspase-9 activation [33]. The active caspase-9 cleaves and activates effector caspases, including caspase-3. The activated effector caspases cleave many structural and functional proteins and activate DNase which destroys chromosomes and leads to cell death [31,32]. As shown with Bax-knock-out mice, ethanol-induced apoptosis in the developing brain is a Bax-dependent process which requires translocation of the Bax protein from the cytosol to mitochondria, disruption of mitochondrial membranes, release of cytochrome C and activation of caspase-3. All these events and also the absence of caspase-8 activation indicate that alcohol induces apoptosis in the developing brain via intrinsic (mitochondrial-mediated) apoptotic pathways [34,35].

In the present experiments we demonstrate that acute ethanol administration to P4 mice induces activation of caspase-3 and DNA fragmentation in Purkinje neurons and rats in all vermian lobes studied. This finding is in concert with the results of other authors working with mice [25] and rats [36]. Ethanol administration to mice pups at age P7 markedly enhanced the number of activated caspase-3-IR and TUNEL-positive cells in the IGL but did not markedly affect the Purkinje cells. This time difference in alcohol sensitivity of different types of neurons is not surprising, being already shown in other studies [25].

The presence of taurine at high concentrations during the early ontogenesis is essential for normal development [37]. Taurine has been tested in treatment of many diseases, including cardiovascular disorders, epilepsy, macular degeneration, hepatic disorders, cystic fibrosis, Alzheimer’s disease and alcoholism [38]. It also interacts with the effects of ethanol [39]. For instance, it modulates ethanol-stimulated locomotion [40] and prolongs ethanol-induced sedation when given intracerebroventricularly to mice [41,42]. Furthermore, ethanol administration elicits an increase in extracellular taurine in the rat cerebral cortex and hippocampus [43]. However, many findings on taurine and ethanol interactions have been contradictory. For instance, in behavioural studies taurine pretreatment has reduced the duration of ethanol-induced sleep-time and attenuated the loss of righting reflex [44,45], not altered the ethanol-induced loss of righting reflex [46] or even enhanced it [41]. It seems that interactions of taurine and ethanol in the brain depend largely on the experimental set-up and the doses of ethanol and taurine administered [19].

Mounting recent evidence indicates that taurine is involved in apoptosis regulation and protects many cell types under different pathological conditions such as ischemia [22,23], high glucose level [47], oxidative stress [48], and ethanol intoxication [49]. In adult rats intraperitoneal taurine injections markedly increase the taurine concentration in blood plasma and brain microdialysates in a dose-dependent manner [50]. After a single injection of 1 g/kg taurine the maximal plasma and brain concentrations are reached within 20 min and they remain significantly higher than in controls for 3.5-4 h. The present two taurine injections at the dose of...
1 g/kg each with the 4-h interval keep the level of taurine high for a more prolonged time. The present finding that in this manner taurine attenuated apoptosis in the IGL is in accordance with our previous study [49].

However, in P4 mice we got unexpected results that taurine had no effect on the ethanol-induced activation of caspase-3 and apoptosis in Purkinje cells. Taurine can be involved in apoptosis regulation by several ways. First of all, taurine decreases intracellular free Ca^{2+} by inhibiting all types of voltage-gated calcium channels and the N-methyl-D-aspartate (NMDA) receptor-gated calcium channel [24,51] and by increasing Ca^{2+} buffering in mitochondria [52]. It prevents in this manner activation of calpain (calcium-dependent protease) and protects mitochondrial membranes from disruption [24]. Taurine can also protect cells acting as antioxidants [15], scavenging at its different physiological concentrations many reactive oxygen and nitrogen species [53], although ethanol administration to neonatal rats may not induce oxidative stress to cerebellar granule neurons [54]. For cell survival a balance between the pro-apoptotic protein Bax and the antiapoptotic protein Bcl-2 is very important. A decrease in the Bcl-2 level in cells leads to translocation of Bax to mitochondria, disruption of their membranes and a release of cytochrome C from mitochondria to the cytosol [55]. Taurine application can restore the pool of Bcl-2 and protect cells against apoptosis [24]. Since ethanol-induced apoptosis is a Bax-dependent process [34], we suggest that restoration of the Bcl-2 level was one possible mechanism of apoptosis prevention in our experiments. Taurine may also be able to rescue cells from apoptosis after the release of cytochrome C from mitochondria. In ischemic cardiomyocytes taurine suppresses the formation of the Apaf-1/caspase-9 apoptosome, prevents caspase-9 activation and thereby preserves cells from apoptosis [22]. All these mechanisms may explain why taurine protects IGL neurons from ethanol-induced apoptosis in P7 mice.

It also seems important to consider another possibility of taurine protection against the adverse effect of ethanol, which is not directly related to apoptosis. Alcohol alters physical properties of membrane lipids, changing membrane fluidity and affecting physiologically important membrane enzymes, for instance, Na^+/K^+-ATPase activity [56-59]. The effects of alcohol on biological membranes depend on the duration of its administration. Acute ethanol administration increases the membrane fluidity [60-62], whereas chronic alcohol intake decreases the fluidity of membranes [60,63,64], which become more tolerant to the disordering effect of ethanol [60,63]. Acute ethanol exposure also decreases Na^+/K^+-ATPase activity in the cerebral cortex and brain stem [65] and kidneys of adult rats [66].

The experimental data on the chronic alcohol effect on Na^+/K^+-ATPase activity seem more contradictory. For instance, chronic alcohol intake has enhanced Na^+/K^+-ATPase activity in rat erythrocyte membranes [58] and in the brain [67]. However, in other studies the activity of Na^+/K^+-ATPase was significantly decreased after chronic alcohol consumption in human erythrocyte membranes [59] and in the brain and cerebellum of rat offspring upon chronic alcohol exposure in utero [68]. Taurine also acts as a membrane stabilizer and restores the depletion of Na^+/K^+-ATPase activity due to ozone exposure and prevents the depletion of the enzyme activity due to cholesterol enrichment [69]. Furthermore, taurine decreases the fluidity of biological membranes when coadministered with calcium [70]. It seems thus possible that taurine in addition to its antiapoptotic actions can reduce the adverse effects of acute ethanol administration acting as membrane stabilizer by the decreasing membrane fluidity and restoring the Na^+/K^+-ATPase activity.

However, the above considerations do not explain why taurine did not affect apoptosis of Purkinje cells on P4. An explanation may be the different functional properties of the neurons studied. The granular neurons in the IGL are glutamatergic and the Purkinje cells GABAergic. Ethanol can induce apoptosis in the developing brain by dual mechanisms, by either blocking NMDA receptors and or by excessively activating GABAA receptors [7]. Taurine itself is an inhibitory amino acid which mimics GABA actions [71,72]. On the other hand, taurine inhibits the glutamate-induced Ca^{2+} elevation [73]. Another simple explanation for different effects of taurine on ethanol-induced apoptosis in Purkinje and IGL cells may be the difference in time when the neuronal populations were studied. We studied P4 mice at 12 hours and P7 mice at 8 hours after the first ethanol administration. Taurine is readily excreted in urine and its plasma concentration decreases during the experiments. At 8 hours the concentration was still relatively high but at 12 hours almost at the control level; it was not high enough to protect Purkinje cells.

Conclusions
We show that acute alcohol administration induces apoptosis in Purkinje cells and in cells in the internal granule cell layer (IGL) of the cerebellum. The time frame of sensitivity to ethanol administration is different for Purkinje and IGL cells. Taurine application was neuroprotective against ethanol-induced apoptosis in cells in the IGL. Prevention of caspase-3 activation and DNA fragmentation by taurine in IGL cells is likely to be due to one or several of the following mechanisms, including the restoration of the pool of Bcl-2, regulation of intracellular Ca^{2+} and inhibition of caspase-9 activation. The
failure of prevention of ethanol-induced apoptosis by taurine in Purkinje cells may result from the different functional properties of the neurons studied. GABAergic Purkinje cells are inhibitory and glutamatergic granular cells excitatory. The decrease in the taurine level during functional properties of the neurons studied. GABAergic

kinje cells were studied after a longer interval than IGL cells excitatory. The decrease in the taurine level during

variance; IGL: internal granular cell layer; IR: immunoreactive; NMDA: N-methyl-D-aspartate.

http://www.jbiomedsci.com/content/17/S1/S12

Page 10 of 11

List of abbreviations used
HPLC: high-performance liquid chromatography; PI: postnatal day 4; P7: postnatal day 7; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; OPA: α-phenalddehyde; ANOVA: analysis of variance; IGL: internal granular cell layer; IR: immunoreactive; NMDA: N-methyl-D-aspartate.

Acknowledgements
The authors are deeply grateful to Mrs. Raija Repo, Mrs. Irma Rantamaa and 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. This article has been published as part of Journal of Biomedical Science Volume 17 Supplement 1, 2010. Proceedings of the 17th International Meeting of Taurine. The full contents of the supplement are available online at http://www.jbiomedsci.com/supplements/17/S1.

Author details
1 Brain Research Center, University of Tampere Medical School, Tampere, Finland. 2 Laboratory of Comparative Somnology and Neuroendocrinology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St.-Petersburg, Russia. 3 Department of Developmental Biology, University of Tampere Medical School and Department of Pathology, Tampere University Hospital, Tampere, Finland. 4 Department of Paediatrics, Tampere University Hospital, Tampere, Finland.

Authors’ contributions
AGF did all laboratory work and drafted the manuscript; EYT performed the statistical analyses and prepared illustrations, IMP participated in invention of the idea and design of the study, MPH was the expert in immunohistochemistry and PS and SSO acted supervisors and composed the final version of this article. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no competing interests.

Published: 24 August 2010

References
Mechanisms of caspase activation.

Brain Res


Pharmacol Biochem Behav

Neuroprotection by taurine in 11:130-3.

Taurine in neurotransmission.


Cite this article as: Taranukhin et al. Neuroprotection by taurine in ethanol-induced apoptosis in the developing cerebellum. Journal of Biomedical Science 2010 17(Suppl 1):S12.

Submit your manuscript to BioMed Central and take full advantage of:

Submit your manuscript at www.biomedcentral.com/submit

• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution
Taurine protects cerebellar neurons of the external granular layer against ethanol-induced apoptosis in 7-day-old mice

a Medical School, University of Tampere, Finland
b Laboratory of Comparative Somnology and Neuroendocrinology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St.-Petersburg, Russia
c Department of Paediatics, Tampere University Hospital, Tampere, Finland

Corresponding author: Mr. Andrey Taranukhin, Medical School, FI-33014 University of Tampere, Finland.
Phone: +358 3 3551 6712, fax: +358 3 3551 6170, E-mail: andrey.taranukhin@uta.fi
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**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>EGL</td>
<td>external granular layer</td>
</tr>
<tr>
<td>FAS</td>
<td>fetal alcohol syndrome</td>
</tr>
<tr>
<td>IGL</td>
<td>internal granular layer</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>P0</td>
<td>day of birth</td>
</tr>
<tr>
<td>P6</td>
<td>postnatal day 6</td>
</tr>
<tr>
<td>P7</td>
<td>postnatal day 7</td>
</tr>
<tr>
<td>P8</td>
<td>postnatal day 8</td>
</tr>
<tr>
<td>P21</td>
<td>postnatal day 21</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling</td>
</tr>
</tbody>
</table>
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, 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 1 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; Taranukhin et al. 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; Taranukhin et al. 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 mmol/l 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 4ºC. 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 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 1000 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 4ºC. 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 H₂O₂ 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 x 75 and x 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 mm² 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 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 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 2- and 3-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\textsuperscript{2+}, restoration of the pool of Bcl-2 and inhibition of caspase-9 activation.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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.

References


Figure legends

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 x 75. Scale bar: 1 mm.

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 x 750. Scale bar: 0.05 mm.

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 mm² with standard deviations. Number of animals in each group is 5 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 mm² with standard deviations. Number of animals in each group is 5 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
Figure 1.
Figure 2.
Figure 3.
Figure 4.
LETHALITY OF TAURINE AND ALCOHOL CO-ADMINISTRATION IN MICE

Andrey G. Taranukhin\textsuperscript{1,2}, Pirjo Saransaari\textsuperscript{1} and Simo S. Oja\textsuperscript{3}

\textsuperscript{1}Brain Research Center, University of Tampere Medical School, Tampere, Finland, \textsuperscript{2}Laboratory of Comparative Somnology and Neuroendocrinology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St.-Petersburg, Russia, \textsuperscript{3}Department of Paediatrics, Tampere University Hospital, Tampere, Finland

Abstract
Alcohol consumption by mothers during pregnancy causes a fetal alcohol syndrome associated with massive neuronal apoptosis. We have recently shown that taurine at a dose of 2 g/kg saves about 50\% of dying cerebellar neurons from ethanol-induced apoptosis in 7-day-old mice. However, a further increase in the taurine dose to ethanol-treated mice had a toxic and in some cases lethal effect. In the present work we studied the toxic effects of taurine and ethanol co-administration in three age groups: 7-day-old, adult (5-6-month-old) and old (12-13-month-old) mice. Taurine and ethanol were injected in two half-doses: taurine at 0 h and 4 h and ethanol at 1 h and 3 h. The minimal 100\% lethal doses in co-administration of taurine and ethanol were the following: 7-day-old mice - 6 g/kg taurine + 5 g/kg ethanol, adult mice - 10 g/kg of taurine + 8 g/kg of ethanol and old mice above 6 g/kg of taurine + 6 g/kg of ethanol. All mice treated with taurine or ethanol alone survived. The adult and old mice dying from the combined toxicity of taurine and ethanol showed a marked fall in blood glucose, which may be one reason for lethality. A comparison of the lethal doses of taurine and ethanol co-administration in different age groups allows us to conclude that the adverse effect of the combined toxicity of taurine and ethanol is age-dependent.
1 INTRODUCTION

Maternal alcohol consumption during pregnancy may give rise to the cause a set of serious childhood health problems known as fetal alcohol syndrome (Eustace et al. 2003) and to alcohol-related birth defects (Warren and Bast 1988), among them growth retardation, structural brain abnormalities, behavioral and cognitive problems, and learning difficulties (Chudley et al. 2005). In rodents (Ikonomidou et al. 2000; Dikranian et al. 2005) and non-human primates (Farber et al. 2010) in vivo and in humans in vitro (Hao et al. 2003) alcohol induces massive death of neurons in the developing brain by apoptosis, a kind of cell death (Hotchkiss et al. 2009), this possibly accounting for the morphological and functional disorders in children prenatally exposed to alcohol. Prevention of ethanol-induced apoptosis can save many neurons and significantly reduce sequences of alcohol intoxication. Among possible drugs for apoptosis prevention taurine seems particularly attractive, since it is a compound naturally present in abundance in the nervous system (Huxtable 1992; Sturman 1993), involved in apoptosis regulation (Takatani et al. 2004; Wu et al. 2009) and protective to many types of cells under different pathological conditions (Ulrich-Merzenich et al. 2007; Taranukhin et al. 2008; Das et al. 2009; Zhang et al. 2010). We have previously shown that taurine at a dose of 2 g/kg saves about 50% of dying neurons from ethanol-induced apoptosis in the internal (Taranukhin et al. 2010) and external (Taranukhin et al. 2012) layers of the developing cerebellum of 7-day-old mice. However, a further increase in taurine doses with an eye to protecting more neurons becomes dangerous for the whole organism and may even kill treated animals. In the present work we describe the new phenomenon of combined toxicity of taurine and ethanol and seek to establish its possible mechanisms.

2 MATERIALS AND METHODS

2.1 Animals and experimental protocols

Adult NMRI mice for experiments and breeding were purchased from Harlan, the Netherlands. In the experiments male and female mice of three age groups: 7-day-old (day of birth is day 0), adult (5-6 month-old) and old (12-13-month-old) mice were used.
The mice were divided into four experimental groups: control, ethanol-treated, taurine-treated and ethanol+taurine-treated. Ethanol (20 % w/v solution diluted in saline) and taurine (7 % w/v solution diluted in saline) were administered subcutaneously to 7-day-old mice and intraperitoneally to adult and old mice. The different doses of ethanol tested ranged from 0 to 12 g/kg and of taurine from 0 to 12 g/kg together with their combinations, the objective being to find a minimal 100 % lethal dose at each age. Taurine and ethanol were injected in two half-doses: taurine at 0 h and 4 h, ethanol at 1 h and 3 h. The control animals were given saline injections equal to those given to the ethanol+taurine-treated group. The animals were monitored for 14 days to detect any signs of toxicity or lethality.

2.2 Measurements of blood glucose

A part of the adult and old mice were used for measurement of blood glucose levels. Deaths of mice used for the glucose assays were not included in calculation of lethality to avoid any possible influence on the rate of lethality arising from the procedure of blood collection and hemorrhage. Blood samples were taken from the tail vein of each animal at two time-points – in adult mice two hours before the first taurine injection to measure the baseline level of glucose and then 0.5 h after the last taurine injection, and in old mice also two hours before the first taurine injection and then 1 or 2 or 3 h after the last taurine injection to assess the effect of ethanol and taurine on blood glucose. This difference in time-points of blood sampling in adult and old mice was adapted to obtain samples maximally close to and immediately prior to death. At each time-point duplicate blood samples (5 µl) were collected into HemoCue Glucose cuvettes and immediately analysed in a HemoCue B-Glucose Analyzer (HemoCue AB, Ångelholm, Sweden).

2.2 Statistical analyses

Data on mouse mortality are expressed in percentages (Figs 1, 2 and 3). Each value for blood glucose is expressed as the mean ± SD. Statistical significance was determined by Student's t-test (for adult mice) and by one-way ANOVA with Tukey-Kramer post hoc test (for old mice). Statistical comparison was made between “Before” and other time points for each experimental group separately. Differences were considered statistically significant at a P value < 0.05.
3 RESULTS

3.1 Combined toxicity of taurine and ethanol in 7-day-old mice

To reduce the possible influence of individual hereditary characteristics on experimental results the mice from each litter were divided into different experimental groups. Each group consisted of an equal number of males and females. As there was no difference in lethality between males and females, we show total data on lethality in both sexes. Taurine at a total dose of 4 g/kg co-administered with ethanol at a total dose of 5 g/kg killed 58 % of 7-day-old mice. An increase in taurine dose to 6 g/kg administered to ethanol-treated mice induced 100 % mortality (Fig. 1). Though we monitored the mice for 14 days after treatment to detect any signs of toxicity, they usually died during the first hours after the last taurine injection. All mice exposed to ethanol or taurine alone and mice treated with 2 g/kg taurine co-administered with 5 g/kg ethanol survived.

![Fig. 1](image)

Fig. 1. Lethality among 7-day-old mice co-administered with taurine and ethanol. Control, n=6; ethanol 5 g/kg, n=6; taurine 6 g/kg, n=6; E+T 2g/kg, n=6; E+T 4g/kg, n=12; E+T 6g/kg, n=12

3.2 Combined toxicity of taurine and ethanol in adult mice

In the first set of experiments on adult (5-6-month-old) mice we established that the total dose of ethanol 8 g/kg administered in two half-doses
Taurine and alcohol lethality

with a 2-hour interval was the maximal dose not lethal for mice of this age (Table 1). Co-administration of taurine at a dose of 8 g/kg with ethanol (8 g/kg) killed 50% of the adult mice. One hundred % lethality was observed at a taurine dose of 10 g/kg administered to ethanol-treated mice (Fig. 2). All mice treated with taurine or ethanol alone at the doses used survived for the 14 days of observation. All those treated with 6 g/kg taurine co-administered with 8 g/kg ethanol likewise survived.

Table 1. Mouse mortality data

<table>
<thead>
<tr>
<th>Ethanol, g/kg</th>
<th>Death/Number in a group</th>
<th>Adult mice</th>
<th>Old mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2/2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2/2</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0/2</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0/2</td>
<td>0/4</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Lethality among 5-6-month-old mice co-administered with taurine and ethanol. Control, n=2; ethanol 8 g/kg, n=2; taurine 10 g/kg, n=2; E+T 6 g/kg, n=2; E+T 8 g/kg, n=2; E+T 10 g/kg, n=5, E+T 12 g/kg, n=2
3.3 Combined toxicity of taurine and ethanol in old mice

In the preliminary experiments on old (12-13-month-old) mice we found the total dose of ethanol 6 g/kg administered in two half-doses with a 2-hour interval to be the maximal dose not lethal for mice of this age (Table 1). Testing different taurine doses co-administered with 6 g/kg of ethanol we found taurine at a dose of 4 g/kg administered to the ethanol-exposed mice to kill 22 % of treated mice. Taurine at a total dose of 6 g/kg combined with 6 g/kg ethanol resulted in 69 % mortality (Fig. 3). All mice treated with taurine or ethanol alone survived the whole 14-day period of observation.

![Graph showing lethality among 12-13-month-old mice co-administered with taurine and ethanol. Control, n=2; ethanol 6 g/kg, n=5; taurine 6 g/kg, n=4; E+T 2 g/kg, n=3; E+T 4 g/kg, n=9; E+T 6 g/kg, n=13.]

3.4 Changes in blood glucose level after taurine and ethanol co-administration in adult mice

Adult mice treated with 10 g/kg taurine combined with 8 g/kg ethanol usually died within 1 h after the last taurine injection. To measure the blood glucose level maximally close to death we assayed it 0.5 h after the
last taurine injection. Two hours before the treatment we measured the basic level of glucose. It was similar in all experimental groups (8.5 ± 0.9 mmol/l), with individual variations from 7.5 to 11.1 mmol/l. Co-administration of taurine and ethanol significantly reduced the glucose level from 9.3 ± 1.6 mmol/l to 5.6 ± 0.7 mmol/l (Fig. 4). Ethanol alone tended to increase blood glucose, but the difference from 8.6 ± 0.2 mmol/l to 12.3 ± 2.1 mmol/l, was not statistically significant by reason of wide variation. Taurine alone did not alter the blood glucose level.

![Graph showing blood glucose levels](image)

**Fig. 4.** Drop in blood glucose in 5-6-month-old mice after taurine and ethanol co-administration. Control, n=3; ethanol 8 g/kg, n=3; taurine 10 g/kg, n=3; E 8 g/kg +T 10 g/kg, n=3.

### 3.5 Changes in blood glucose level after taurine and ethanol co-administration in old mice

Old mice treated with 6 g/kg of taurine and 6 g/kg of ethanol died within 3-4 hours after the last taurine injection. We measured blood glucose at 1, 2 and 3 hours after the last taurine injection to monitor changes in blood glucose prior to death. Two hours before the treatment the basic level of glucose was similar in all experimental groups (8.5 ± 1.2 mmol/l), with individual variations ranging between 6.9 and 11.2 mmol/l. Taurine and ethanol co-administration significantly lowered the glucose level from 8.5 ±
1.3 mmol/l to 6.1 ± 1.0 mmol/l at 1 h after the last taurine injection. This decrease was preserved for the next two hours, being 4.3 ± 2.0 mmol/l before the death of the animals (Fig. 5). The high SD (2.0 mmol/l) at this last time-point results from substantial differences in blood glucose levels in mice treated with ethanol and taurine. The individual differences varied between 2.2 and 6.0 mmol/l. Taurine alone lowered blood glucose in the old mice from 8.8 ± 1.0 mmol/l to 5.6 ± 1.8 mmol/l in 1 h after the last taurine treatment. The blood glucose level increased slightly during the next two hours to reach almost to the level before experiments. Ethanol alone did not affect the blood glucose level.

**Fig. 5.** Drop in blood glucose level in 12-13-month-old mice after taurine and ethanol co-administration. Control, n=3; ethanol 6 g/kg, n=4; taurine 6 g/kg, n=5; E 6 g/kg +T 6 g/kg, n=5.

### 4 DISCUSSION

Taurine (2-aminoethanesulphonic acid) is a sulfur-containing amino acid abundant in electrically excitable tissues such as the brain, retina, heart and skeletal muscles (Lourenco and Camilo 2002). It is involved in a wide range of physiological processes such as osmoregulation, lipid metabolism, intracellular calcium regulation, neuronal development, neuromodulation and cell protection (Huxtable 1992; Saransaari and Oja 2000; An-
Taurine and alcohol lethality

Growing evidence indicates that taurine is also involved in the regulation of apoptosis (Takatani et al. 2004; Ulrich-Merzenich et al. 2007; Taranukhin et al. 2008; Wu et al. 2009). Clinically taurine has been tested for the treatment of a variety of diseases, including for example alcoholism (Birdsall 1998). Previously, we successfully used taurine at a dose 2 g/kg to save about 50 % of dying neurons from ethanol-induced apoptosis in the developing cerebellum of 7-day-old mice (Taranukhin et al. 2009, 2010 and 2012). We now increased the taurine dosage two- and three-fold in an attempt to obtain more significant benefits in cell protection against alcohol-induced apoptosis. However, contrary to our expectations taurine at a dose of 4 g/kg co-administered with 5 g/kg ethanol killed about 60 % of treated mice and 6 g/kg combined with 5 g/kg ethanol 100%. Interestingly, 6 g/kg taurine and 5 g/kg ethanol alone were safe for the animals. It would thus appear that taurine potentiates the adverse effects of ethanol.

It is well known that ethanol at high doses can induce death, but little is known of taurine toxicity or of the combination of taurine with ethanol or other drugs. In experiments on dogs taurine at a single intravenous dose of 2 g/kg has proved safe for the animals and during a 14-day period of observation there were no dead animals or any signs of toxicity (Nishizawa et al. 1991). Experiments on Wistar rats have shown that taurine in a single dose of 7 g/kg (intravenously) or 5 g/kg (orally) has no toxic effect on the animals, which also survived for 14 days without any sign of toxicity or abnormality upon autopsy (Kihara et al. 1991). All these studies show that taurine is minimally toxic. We found only one study in which taurine at doses of 7.5 g/kg and 6.0 g/kg involved 100 % and 50 % lethality, respectively, after a single subcutaneous injection (Goldberg and Jefferies 1946). In our experiments taurine alone administered at a dose of 6 g/kg was not toxic to the animals, possibly because we injected it in two half-doses 4 h apart. When the animals received first only 3 g/kg taurine and after 4 hours again 3 g/kg, taurine from the first injection was partly incorporated into the bile or secreted into the urine, as shown by the decrease in taurine levels in the blood (Lallemand and De Witte 2004; Taranukhin et al. 2010).

To test the hypothesis that the lethality of combined taurine and ethanol not only pertains for developing 7-day-old mice, which at this age are highly sensitive to ethanol toxicity (Ikonomidou et al. 2000), we repeated our experiments on adult (5-6-month-old) and old (12-13-month-old) mice. We thus established the maximal doses of ethanol for adult (8 g/kg) and old (6 g/kg) mice which do not induce mortality. Our present data (8 g/kg) differ slightly from the results of some other authors (Schechter and Meehan 1995) with regard to the ethanol dose which does not induce death in young adult mice (6 g/kg), probably by reason of differences in ethanol
sensitivity between the two strains of mice tested. Our data on differences in the toxicity of different doses of ethanol in adult and old mice are similar to those obtained on rats, in which the 50% lethal dose for 3-4-month-old rats was significantly higher than that for 10-12-month-old rats (Wiberg et al. 1970). In adult and old mice the combined toxicity of taurine and ethanol sufficient to induce 100% mortality was significantly higher than in 7-day-old mice. Sensitivity to the combined toxicity of taurine and ethanol is thus age-dependent.

Taurine treatment significantly attenuates the stress-induced elevation in blood glucose in rats (Nakagawa and Kuriyama 1975). Taurine exerts a hypoglycemic action in glucose supplementation-induced hyperglycemia (Kulalowski and Maturo 1984; Kaplan et al. 2004). These findings on the ability of taurine to reduce blood glucose and the observation that ethanol can also lower it (Huang and Sjöholm 2008) allows us to assume that the lethality of taurine and ethanol co-administration can be induced by hypoglycemia. Blood glucose after taurine and ethanol co-administration was significantly decreased in adult and old mice. In adult mice the decrease in glucose concentration was still far from the dangerous level. The old mice could be divided into two subgroups, one of which had blood glucose about 6 mmol/l and the other 2.2 mmol/l, which is very low and may be the reason for death. We would suggest that these two subgroups could explain why only 70% of the taurine- and ethanol-treated mice died. The remaining 30% may have had a less dangerous level of blood glucose (6 mmol/l).

5 CONCLUSION

In summary, our finding would indicate that taurine alone administered even at high doses is safe for mice. In contrast, a combination of high doses of taurine with alcohol has a toxic effect and will kill the animals. This lethal effect of taurine and ethanol co-administration may be related to a dramatic drop in blood glucose. Comparison of the lethal doses of taurine and ethanol co-administration in 7-day-old, adult and old mice allows us to conclude that the adverse effects of this combined toxicity is age-dependent. The present findings constitute a serious warning of the toxic interactions of taurine and ethanol particularly for young people mixing taurine-containing energy drinks with alcohol.
6 ACKNOWLEDGEMENTS

This study was supported by the competitive research funding of the Pirkanmaa Hospital District, the Maud Kuistila Memorial Foundation and the Finnish Foundation for Alcohol Studies. We thank Dr. Tiina Solakivi who offered invaluable help with blood glucose determinations.

7 REFERENCES

anol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056-1060

