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ORIGINAL CONTRIBUTION

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Can megadoses of thiamine prevent ethanol induced damages of rat hippocampal CA1 pyramidal neurones?

Können Thiamin-Megadosen alkoholinduzierte Schäden an den CA1 Pyramidenzellen des Hippocampus von Ratten verhindern?

Summary The specific aim of this study was to evaluate whether high doses of thiamine can compensate or prevent alcohol-induced damages of rat hippocampus CA1 pyramids. Twenty weeks of ethanol consumption together with a dose of thiamine in the range of 1.19 mg/100 mg food induced significant enlargement (parameters measured were length of the whole spine and diameter of the endbulb) of dendritic spines. Hypertrophy can be interpreted as a compensation process due to alcohol-induced cell death because viable spines are in search of new synaptic contacts. In contrast, dendritic spines of the alcohol group fed at the same time with a high dose of thiamine (119 mg/100 g food = megavitamintherapy) showed normal data concerning these parameters. From these results it may be concluded that a megavitamin therapy supports a neuron's carbohydrate metabolism and therefore could be able to prevent or reduce alcohol-induced damages of hippocampal CA1 pyramidal cells in rat central nervous system.

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Zusammenfassung In einer 5monatigen Versuchsperiode wurden ethanolinduzierte Schäden an den dendritischen Dornen der CA1-Pyramidenzellen des Hippocampus männlicher Wistarratten auf morphologischer Ebene dokumentiert. Die zentralnervösen Alterationen spiegeln sich nach täglicher Ethanolapplikation mit dem Trinkwasser und Thiamingaben in Höhe der 3fachen alimentären Empfehlung (1,19 mg/100 g Futter) in einer signifikanten Dornenvergrößerung (gemessene Parameter: Dornenlänge und Durchmesser des Dornenkopfes) wider. Die Dornenhypertrophie ist als neuronaler Kompensationseffekt zu interpretieren, der als Folge eines alkoholbedingten Zelltodes auftritt: Überlebende Neurone protrahieren ihre Dornen auf der Suche nach neuen synaptischen Kontakten zur Erhaltung des ‚neuronalen Kreislaufs'. Demgegenüber weisen die dendritischen Dornen alkoholbehandelter Tiere, denen gleichzeitig hochdosierte Thiamingaben (119 mg/100 g Futter) verabreicht wurden, normale Längen und Durchmesser ihrer Endköpfe auf. Möglicherweise kann Thiamin durch die Aufrechterhaltung des neuronalen Kohlenhydratstoffwechsels alkoholinduzierte Degenerationen am zentralen Nervensystem der Ratte verhindern.

Key words Hippocampus – CA1 pyramidal neurone – spines – ethanol – thiamine

Schlüsselwörter Hippocampus – CA1 Pyramidenzelle – dendritische Dornen – Ethanol – Thiamin

Introduction

The hippocampal formation of rodents is particularly sensitive to alcohol (5, 9, 11, 28, 30, 31, 38, 39). Chronic alcohol consumption reduces the number of hippocampal neurones (10, 38) and the complexity of the dendritic arborization (12, 27). Furthermore, often described morphological damages of chronic ethanol treatment are de-

creased spine densities of CA1 hippocampal pyramids (25, 29, 33, 39).

In addition, studies at the ultrastructural level demonstrate that the organization of mossy fibers and the mossy fiber-CA3 synapses, providing a direct connection between the dendate gyrus granule cells and the pyramidal cells of the field CA3 are altered after exposure to ethanol (10, 41, 42).

In the causation of the effects of alcohol on neurones thiamine deficiency plays an important role: chronic alcoholism leads to a reduced supply of essential vitamins, particularly thiamine, causing a reduction of thiamine dependent enzymes involved in brain glucose metabolism (7, 34). Based upon these observations, the purpose of the present study was to examine whether megadosed thiamine supply is able to prevent alcohol induced damages in the rat central nervous system.

Material and methods

Animals and diets

Six-week-old male Wistar rats, weighing a mean of 65 g (breeder: F. Winkelmann, Borchen, FRG), were used in this study. All animals were maintained throughout the experiment under a light/dark cycle of 12/12 h and a room temperature of 20–22 °C. The animals were separated into four groups (CW, CE, TW, TE) of 12 animals. Each animal was individually caged. The alcohol-fed groups CE and TE were given unrestricted access to a 20 % aqueous ethanol solution within 5 % sucrose and were fed a semisynthetic casein diet plus thiamine in the range of 1.19 mg/100 g food (CE) and of 119 mg/100 g food (TE). Animals of the groups CW and TW were given the same amount of fluid and food where sucrose isocalorically replaced the ethanol-sucrose solution, as food and fluid intake (see Table 1) were measured daily and the consumed amounts were calculated.

Histological procedures – Golgi-Impregnation

The animals were deeply anesthetized with a mixture of Ketamin and Rompun and euthanized before the brains were removed. Tissue blocks from the hippocampus formation were immediately fixed by immersion in 8 % formaldehyde solution for 2 weeks and treated according to the Golgi-Bubenaite method (35). For this impregnation-staining tissue blocks were emerged in a solution of 2.5 % potassium dichromate at 34 °C for 48 h, then they were transferred in 2 % silvernitrate. After the impregnation and subsequent dehydration with ehtanol the blocks were embedded in paraffin and cut in 50 µm-thick microtomed sections. These were observed under light microscope and photographed (Axiophot-microscope, Zeiss) for comparative analysis.

Quantitative procedures and statistical analysis

Photographs at a primary magnification of x400 were taken from a Zeiss photomicroscope (Axiophot, Zeiss). The length, observed at its maximum extent, and the diameter of the bulbous end of the dendritic spines were used from negatives under a ‚Meßprofilprojektor' at a final magnification of x10 000 and were obtained by measuring a minimum of 5 CA1 pyramidal neurones (n = 60 for each animal).

Table 1 A general view of food and fluid consumption of the groups CW, TW, TE, and CE during a treatment period of 20 weeks

	Treatment			
Unlimited amount of	**CW** Control diet Without ethanol	**CE** Control diet plus Ethanol	**TW** Thiamine diet Without ethanol	**TE** Thiamine diet plus Ethanol
Food[a]	1.19 mg thiamine per 100 g food ~ 4.5 g sucrose/ 11 g food/animal/ day[b]	1.19 mg thiamine per 100 g food	119 mg thiamine per 100 g food ~ 4.4 g sucrose/ 11.5 g food/animal/ day[b]	119 mg thiamine per 100 g food
Fluid	water	20 % aqueous ethanol solution within 5 % sucrose	water	20 % aqueous ethanol solution within 5 % sucrose

[a] Semisynthetic casein diet for rats plus thiamine.
Sucrose isocallorically replaced the ethanol in the nonalcoholic groups CW and TW.

[b] Sucrose addition, calculated daily for all animals of the groups CW and TW was related to the ethanol consumption of the groups CE and TE per animal and day.

The criteria for cell selection were that the cell and its dendritic network be completely impregnated, undamaged, unobscured, and contained entirely within the section. A limitation of the analytical methods used in this study was that within each group a various number of animals, whose neurones satisfied the above criteria were found, because the impregnation technique of Golgi randomly picked out only a small proportion of neurones present (or only parts of them). Thus, measurements could be made only from a limited number of animals (compare Tables 2 and 3).

The data were tested for statistical significance (ANOVA). An alpha level of 0.05 was used to reject the null hypothesis. Post-hoc Scheffé-tests were applied to cell data which yielded a significant F-value lower than 0.01 to determine the locus of an effect.

Results

Animals and diets

The average alcohol consumption of group CE and TE was approximately 12 g/kg body weight daily. The mean food consumption per animal of the groups CW (10.9 ± 1.5 g/day) and TW (11.4 ± 1.2 g/day) was significantly higher when compared to the alcohol-fed groups CE (6.7 ± 1.7 g/day) and TE (6.5 ± 1.8 g/day). The mean thiamine intake was 0.08 ± 0.02 mg (CW), 0.08 ± 0.02 mg (CE), 9.0 ± 1.87 mg (TW) and 7.77 ± 2.1 mg (TE) per animal and day. No statistically significant differences were found in the mean body weights during the experiment, although alcohol-fed animals of group CE showed a mean body weight increase of 13.5 ± 1.3 g/month and group

Table 2 Quantification of the effects of ethanol exposure and a simultaneous thiamine megavitamin therapy on the length and the diameter of dendritic spines of hippocampal CA1 pyramidal neurones (mean ± SD)

	Treatment[a]			
	Control diet		Thiamine diet	
Animal code number	CW	CE	TW	TE
# 1	0.37 ± 0.12	[c]	0.41 ± 0.09	0.41 ± 0.10
	0.35 ± 0.07	[c]	**0.36 ± 0.08**	**0.41 ± 0.09**
# 2	[c]	[c]	0.41 ± 0.10	0.46 ± 0.10
	[c]	[c]	**0.35 ± 0.07**	**0.40 ± 0.10**
# 3	0.39 ± 0.12	[c]	0.39 ± 0.08	0.46 ± 0.09
	0.32 ± 0.07	[c]	**0.39 ± 0.13**	**0.42 ± 0.10**
# 4	0.37 ± 0.10	0.76 ± 0.19	0.41 ± 0.12	[c]
	0.40 ± 0.08	**0.49 ± 0.08**	**0.35 ± 0.06**	[c]
# 5	0.38 ± 0.10	0.70 ± 0.21	0.43 ± 0.09	0.42 ± 0.09
	0.35 ± 0.09	**0.49 ± 0.09**	**0.35 ± 0.07**	**0.41 ± 0.09**
# 6	0.43 ± 0.11	0.75 ± 0.18	0.42 ± 0.11	0.40 ± 0.08
	0.35 ± 0.07	**0.53 ± 0.02**	**0.37 ± 0.07**	**0.41 ± 0.08**
# 7	0.43 ± 0.09	0.81 ± 0.17	0.37 ± 0.08	[c]
	0.39 ± 0.09	**0.49 ± 0.02**	**0.33 ± 0.06**	[c]
# 8	0.40 ± 0.13	0.80 ± 0.16	0.39 ± 0.07	0.45 ± 0.11
	0.40 ± 0.09	**0.49 ± 0.11**	**0.36 ± 0.07**	**0.39 ± 0.07**
# 9	0.39 ± 0.09	0.78 ± 0.16	0.42 ± 0.10	0.43 ± 0.10
	0.34 ± 0.07	**0.51 ± 0.10**	**0.33 ± 0.08**	**0.43 ± 0.08**
# 10	0.38 ± 0.09	0.69 ± 0.15	0.39 ± 0.11	[c]
	0.36 ± 0.08	**0.54 ± 0.12**	**0.34 ± 0.07**	[c]
# 11	0.39 ± 0.10	0.77 ± 0.19	[c]	0.45 ± 0.09
	0.36 ± 0.07	**0.46 ± 0.11**	[c]	**0.41 ± 0.09**
# 12	0.39 ± 0.10	0.77 ± 0.17	0.40 ± 0.09	[c]
	0.34 ± 0.06	**0.48 ± 0.16**	**0.36 ± 0.06**	[c]
Total Mean ± SD[b]	0.39 ± 0.02	0.76 ± 0.04	0.40 ± 0.02	0.44 ± 0.02
	0.36 ± 0.03	**0.50 ± 0.02**	**0.35 ± 0.02**	**0.41 ± 0.01**

[a] treatment groups: CW, CE, TW, TE (compare Table 1)
[b] all measurements in micrometers
spine length: standard font
spine diameter: bold and italic
[c] lacking measurements because of non-impregnation of the specimens

Table 3 Significant differences of dendritic spine length carried out by using the post-hoc Scheffe-test.

	Treatment groups[a]			
	Control diet		Thiamine diet	
	CW	CE	TW	TE
Total mean ± SD[b]	0.39 ± 0.02 **0.36 ± 0.03**	0.76 ± 0.04 **0.50 ± 0.02**	0.40 ± 0.02 **0.35 ± 0.02**	0.44 ± 0.02 **0.41 ± 0.01**
CW		*	n.s.	*
		♦	**n.s.**	♦
CE			*	*
			♦	♦
TW				*
				♦

[a] treatment groups: CW, CE, TW, TE (compare Table 1)
[b] all measurements in micrometers
spine length: standard font
spine diameter: bold and italic
* significant concerning length
♦ significant concerning diameter

TE, 11.4 ± 1.6 g/month, whereas the weights of the rats of experimental group CW revealed an average increase of 17.1 ± 1.4 g and TW of 18.8 ± 1.7 g per month.

Histology of Golgi-stained neurones

Light microscopic observation of Golgi-impregnated CA1 pyramidal neurones of the groups CW, TW and TE (Fig. 1 a–b) showed spines (n = 60 for each animal) of normal appearance. The apical and basal spiny branchlets as overviewed in Fig. 1a were uniformly crowded and spines could be morphologically characterized as the typical "stubby-shaped" protrusions, which are represented in Fig. 1b.

In contrast, following 20 weeks of ethanol consumption together with dosages of thiamine in the range of 1.19 mg/100 g food (CE) spines of the apical and basilar dendrites appeared remarkably altered. There was a predominance of dendritic spines with long stalks and prominent round end-bulbs, whose morphology is demonstrated in Fig. 1c.

Statistical analysis

The data concerning dendritic spine lengths and diameters of the end-bulbs of hippocampal CA1 neurones are summarized in Table 2. There were significant main effects of spine size due to ethanol treatment. The post-hoc Scheffé-test documented that 5 months of ethanol treatment together with a dose of thiamine in the range of 1.19 mg/100 g food resulted in significantly elongated spines and in significant increases of the diameters of the spiny end-bulbs (Table 3), whereas spines from alcohol-fed rats on the thiamine containing megatherapy

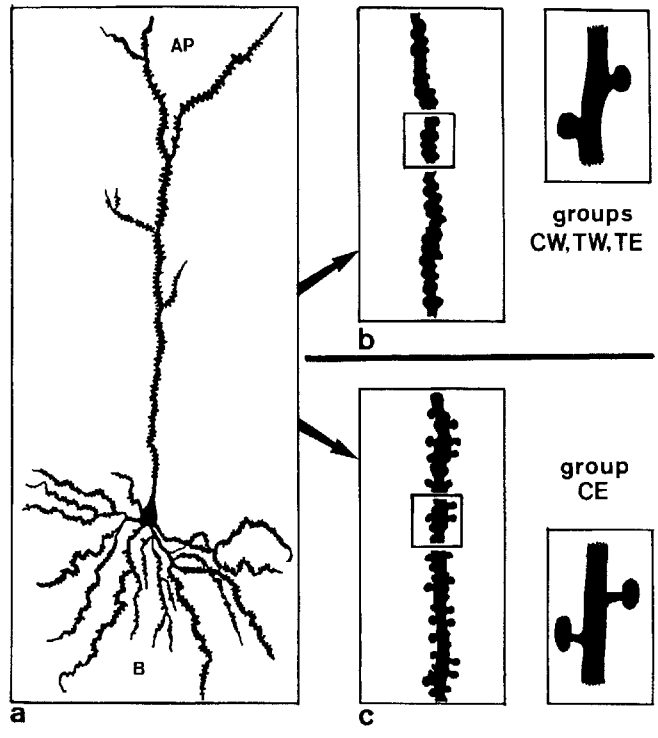

Fig. 1 A–C Drawing of a Golgi-impregnated rat CA1 pyramidal cell at subsequent higher magnifications. **A** A Golgi-impregnated CA1 pyramidal cell with its apical (AP) and basilar (B) dendrites, studded uniformly with spines (represented as small protrusions along the dendritic processes). **B** An apical or basilar Golgi-stained dendrite of normal appearance, showing typical "stubby-shaped" spines characterizing the undamaged basilar and apical dendrites of CA1 rat pyramidal cells of the groups CW, TW, and TE. **C** Changed, alcohol-induced morphology of an apical or basilar dendrite of an alcohol-fed rat of the group CE (compare B) whose spines are characterized after long-term alcohol consumption by a long, thinly-necked round end-bulb.

(119 mg/100 g food) and from the groups CW and TW were normal in length and in diameter of the end-bulb related to these parameters.

Discussion

Methodological aspect

Although the investigation were performed on rats which were at the beginning of the main growth phase when the experiment starts data derived from developmental studies demonstrate that rat hippocampal pyramidal cells are generated over relatively short prenatal periods and differentiation processes occur only up to the first postnatal week (4). Therefore, the cytomorphological and cytoarchitectural features of the hippocampal formation correspond to the pattern of adult rats when the treatment period starts and results obtained can be applied to adult rats.

The data documented in the present study indicate for the first time that long-term ethanol application induces spine hypertrophy of rat CA1 hippocampal pyramids. The method of Golgi-staining was highly selective and has provided more information about the morphological details of the dendritic arborization of a CA1 neurone than any other staining, and therefore was selected as the adequate staining procedure here and elsewhere (37). This is vulnerable even though some measurements of each animal group were lacking (compare material and methods, and Table 2), because the Golgi-staining randomly impregnated only a small proportion of neurones present. Therefore, cell count studies which are necessary for the interpretation of neuronal cell death which possibly occurred cannot be made with this histological method.

Spine hypertrophy – a compensation process in response to ethanol

Tavares et al. (37) report elongation of Golgi-impregnated Purkinje cell dendritic spines to 3 or 4 times their normal length after alcohol treatment. These findings support the view that neurones probably compensate for alcohol-induced damages, not only in the cerebellum (40), but also in the hippocampus in such a manner that ethanol causes changes and loss of a large number of neurones, whereas viable cells show enlargement of intact spines which are in search of new synaptic contacts maintaining the neuronal circuit.

The hippocampus – a vulnerable brain region

The hippocampal formation receives an important cholinergic innervation from the nuclei of the basal forebrain which are severely vulnerable to ethanol (2, 3, 13). The effects of ethanol within these brain areas cause a reduced cholinergic input to the hippocampus formation and give rise to the reduction of density of hippocampal choline acetyltransferase reactive fibers. Hippocampal CA3 and CA1 pyramidal cells are the targets of cholinergic inputs and therefore it may be assumed that changes of the neuronal circuit are a consequence of alterations in hippocampal cholinergic afferents, inasmuch as alcohol *per se* – induces degeneration of the very same neurone. This might be more the result of a decreased muscarinic receptor density (11).

Effects of ethanol in the brain

Behavioural (32) and neurochemical (15, 21, 24, 44) effects of ethanol administration are dose-dependent. Williams-Hemby and Porrino (43) evaluate ethanol-induced, dose-dependent changes in cerebral glucose metabolism in different brain regions of the rat with the 2-(14 C) deoxyglucose (2-DG) method. The authors have demonstrated that higher doses of ethanol which were applied in the present study in the range of 12 g/kg body weight daily (Blood ethanol levels have not been measured) result in decreases of local cerebral glucose utilization (LCGU) in several brain areas including the hippocampal complex and its anatomically related regions whereas low doses (0.25–0.5 g/kg) induced an increased glucose metabolism, particularly in the mesocorticolimbic dopamine system.

The metabolic changes within the hippocampal complex correspond to the effects of ethanol on cell firing rates in the hippocampus (17, 26). Increases can be detected at low doses and decreases are seen at higher doses. Therefore, it may be concluded that metabolic changes in glucose metabolism and in functional activity mediate some of the intoxicating effects of ethanol seen in the present study. Regardless of the dose-dependent regional effects of ethanol, it has been pointed out that the selective topographic vulnerability of brain structures in response to ethanol has been based upon the expression of particular subunit combinations of ligand-gated ion channels ($GABA_A$, NMDA, 5 HT3 receptors; 16). Furthermore, it has been proposed that a region-selective loss of thiamine diphosphate, causing reductions in activities of thiamine dependent enzymes, plays a major role in the pathogenesis of neuronal cell loss (6).

Alcohol-induced brain damage possibly also involves spasm of cerebral blood vessels causing hypoxia (1), fluidisation of membranes (37), and focal accumulation of aldehyde (23).

Causation of the effects of ethanol in relation to thiamine deficiency

Neuromorphological and neurofunctional damages of alcoholism such as described above often originate from interactive factors involving direct nervous system tox-

icity of ethanol *per se* (18, 22) and nutrient deficiencies associated with heavy drinking. In the latter case a reduced supply of essential vitamins, particularly thiamine, has been documented as caused by inadequate dietary intake of vitamins, impaired absorption from the gastrointestinal tract, depletion of liver and brain stores (6) and reduction in thiamine phosphorylation to thiamine pyrophosphate in the brain (34).

Thiamine plays an important role in metabolism of carbohydrates, the major source of which is glucose, because it acts in form of TPP as coenzyme of pyruvate oxidase (pyruvate dehydrogenase complex, PDHC), as coenzyme of transketolase (TK) in the pentose phosphate cycle, and third in the citric acid cycle (α-ketoglutarate dehydrogenase complex, αKGDH). Thus, brain cells require a continuous supply of thiamine (7) and ethanol induced neuronal thiamine deficiency could result in low metabolism rates causing neuronal alterations. Further studies concerning measurements of blood and tissue thiamine levels will clarify the *in vivo* ethanol-thiamine interaction in the rat's metabolism.

Furthermore, pyrithiamine induced thiamine deficiency results in reductions in activity of αKGDH and terminates in neuronal cell death (19). Selective reductions of αKGDH are accompanied by reductions of tricarboxylic acid related aminoacids such as glutamate, aspartate and GABA (7, 8), by diminished pyruvate oxidation (14) and increased alanine (8) and lactate (20). Accumulation of lactate results in pH-changes and acidosis causing an impaired brain energy metabolism which could be responsible for neuronal damages and cell death.

These biochemical data reflect the important role of thiamine in a neuron's metabolism. Therefore, it may be concluded that ethanol-induced damages on CA1 pyramidal neurones, as demonstrated on the morphological level in the present study, could be reduced or prevented as a consequence of a thiamine-support brain metabolism in the rat central nervous system.

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