ELSEVIER

Pharmacology, Biochemistry and Behavior 70 (2001) 227–235

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

The contribution of mild thiamine deficiency and ethanol consumption to central cholinergic parameter dysfunction and rats' open-field performance impairment

Rita G.W. Pires[a], Silvia R.C. Pereira[b], José Eymard H. Pittella[c], Glaura C. Franco[d], Carmencita L.M. Ferreira[c], Paula A. Fernandes[c], Angela M. Ribeiro[a,*]

[a]*Laboratório de Neuroquímica, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biologicas, Universidade Federal de Minas Gerais, 31270-010 Belo Horizonte, Minas Gerais, Brazil*

[b]*Departamento de Psicologia, FAFICH, Universidade Federal de Minas Gerais, 31270-010 Belo Horizonte, Minas Gerais, Brazil*

[c]*Departamento de Anatomia e Medicina Legal, Faculdade de Medicina, Universidade Federal de Minas Gerais, 31270-010 Belo Horizonte, Minas Gerais, Brazil*

[d]*Departamento de Estatística, ICEX, Universidade Federal de Minas Gerais, 31270-010 Belo Horizonte, Minas Gerais, Brazil*

Received 14 July 2000; received in revised form 14 May 2001; accepted 14 June 2001

Abstract

We studied at the biochemical, morphological, and behavioral levels the effect of chronic ethanol consumption, associated or not with a mild thiamine deficiency episode. We found that (i) thiamine deficiency induced a significant decrease of the acetylcholinesterase (AChE) activity both in cortex and hippocampus; (ii) chronic ethanol treatment has no effect on cortical AChE activity, but induced a significant decrease of hippocampal enzyme activity; (iii) the reduction in cortical and hippocampal AChE activity induced by chronic ethanol treatment associated with a 1-week thiamine deficiency was also significant and was greater than that induced by ethanol alone. Furthermore, either chronic ethanol or thiamine deficiency induced a significant decrease in the release of acetylcholine (ACh) in the stimulated condition using high potassium concentration; and when both treatments were associated the decrease was even greater. In the unstimulated condition, the reduction in the release of ACh was greater for ethanol treatment than for thiamine deficiency. Open-field tests showed that only in the "sniffing" category were there significant differences among the experimental groups. No morphological change was detected by optical microscopy, suggesting that the injury process was in its initial stages in which only functional and behavioral changes are displayed. In addition, our biochemical results indicate that cortical cholinergic susceptibilities to ethanol and thiamine deficiency are significantly different.

Keywords: Ethanol; Thiamine deficiency; Acetylcholinesterase activity; Acetylcholine release; Cerebral cortex; Hippocampus; Open-field; Wistar rat

1. Introduction

Chronic ethanol consumption associated with malnutrition (shortage of thiamine, a vitamin of the B complex) can cause the Wernicke–Korsakoff's syndrome (WKS) (Harper, 1983; Kril, 1995; Pittella and Giannetti, 1994). The conventional idea of the early 1970s was that WKS developed as a result of thiamine deficiency, and that ethanol per se had no direct deleterious effects on the brain. The subsequent demonstration in animal and human neuroradiological and neuropsychological studies that ethanol adversely affects brain structure and function, probably due to direct neurotoxicity, has led to the reevaluation of traditional concepts (Ron et al., 1982). However, it is still unknown whether the cerebral damage seen in chronic alcoholism is caused by one of these factors or by the association of both (Martin et al., 1993). There are contradictory findings on the correlation between chronic ethanol consumption and cognitive impairment (Arendt et al., 1989; Blokland et al., 1993; Casamenti et al., 1993; Pereira et al., 1998). In other studies using animal models, both ethanol consumption and thiamine deficiency alone caused similar and mild damage, while the com-

* Corresponding author. Tel.: +55-31-499-2642; fax: +55-31-441-5963.

E-mail address: angelamr@mono.icb.ufmg.br (A.M. Ribeiro).

0091-3057/01/$ – see front matter
PII: S0091-3057(01)00593-7

bination of these treatments led to more pronounced neurological damage (Palencia et al., 1994). On the other hand, some results indicate that the interaction of chronic ethanol consumption and bouts of thiamine deficiency is not always synergistic. When rats are submitted to both treatments, some behavioral aspects appear to be sensitive to synergistic interaction between ethanol and thiamine deficiency, whereas others are most affected by ethanol; and neurological symptoms are mostly associated with thiamine deficiency (Ciccia and Langlais, 2000). Moreover, it has been shown that common and separate mechanisms underlie the effects of alcohol intoxication and thiamine deficiency on cell death and cell atrophy in hippocampus CA3 pyramidal cells (Bâ et al., 1999). It has been demonstrated that thiamine deficiency can cause behavioral alterations ranging from mild to severe cognitive impairment (Langlais and Savage, 1995). These models produce features similar to its human analogue and it was suggested that a chronic, but subclinical episode of thiamine deficiency might be responsible for pathological changes in the brains of alcoholics (Harper, 1983; Homewood et al., 1997; Lishman, 1981). Loss of cortical neurons has been reported in animals submitted to treatment with ethanol and thiamine deficiency (Kril and Homewood, 1993).

The brain's cholinergic neurons appear to be particularly vulnerable to ethanol (Arendt et al., 1988a,b; Cadete-Leite et al., 1995; Hodges et al., 1991; Pelham et al., 1980; Rawat, 1974; Smyth and Beck, 1969). After intake interruption, the cholinergic hypofunction and behavioral impairment induced by ethanol in rodents is either partially or fully reversible, depending on the duration of both the treatment and the abstinence (Arendt et al., 1989; Casamenti et al., 1993; Floyd et al., 1997). For instance, there is evidence that cholinergic parameters assessed 1 week after withdrawal were significantly lower in ethanol-treated rats while after a period of four ethanol-free weeks, both nonsignificant (Casamenti et al., 1993; Pereira et al., 1998) and significant (Arendt et al., 1989) differences in these parameters between treated and untreated rats were found.

Using an animal experimental model, our research group showed that after a 2-month abstinence there were no significant differences in brain cortical acetylcholinesterase (AChE) activity and stimulated acetylcholine (ACh) release between chronically treated ethanol and control subjects. However, rats chronically treated with ethanol showed a retrograde amnesia, which does not seem to be linked to a cortical cholinergic deficit. In these chronically treated rats, we did not find differences in blood thiamine level between animals from control and treated groups (Pereira et al., 1998).

Regarding the effect of thiamine deficiency on cholinergic parameters, an early functionally significant cholinergic brain impairment related to behavioral change was found (Barclay et al., 1981), while other authors have not found any alterations in muscarinic cholinergic function (Rao et al., 1995). There is evidence of a decrease in ACh metabolism associated with thiamine deficiency, but the real meaning of this cholinergic change remains obscure. Animal models submitted to a thiamine-deficient diet presented impairment in behavior tests, which may reflect an alteration in ACh metabolism (Barclay et al., 1982). Furthermore, in thiamine-deficient animals a significant reduction in ACh content is seen in the striatum, but no alterations are seen in cortex, cerebellum, and hypothalamus (Plaitakis et al., 1982). A decrease in ACh synthesis in vivo and in vitro has also been observed (Gibson et al., 1982). The authors argue that whole brain ACh synthesis and levels may not accurately reflect localized changes in metabolism, and regional studies are clearly needed.

In the central nervous system, forebrain cholinergic projection has two branches: one projects into the hippocampal formation from cells of both nuclei of the medial septal and the diagonal band. The other projects to all areas of the neocortex and to the amygdala from the basal nucleus (Mesulam et al., 1983).

The cholinergic system seems to be involved in open-field test performance. It has been shown that some open-field behavior categories are affected by cholinergic drugs (Barclay and Gibson, 1982; Barclay et al., 1982); that patterns of change in the open-field behavior are correlated to different cholinergic manipulations (Ogishima et al., 1992); and that septal and diagonal band cholinergic lesions affect open-field test performance (Torres et al., 1994).

There is evidence that prolonged ethanol consumption leads to a substantial reduction in the cholinergic innervation of the hippocampal formation but not in that of the neocortex (Cadete-Leite et al., 1995). Although the issues regarding the effects of chronic ethanol consumption alone and thiamine deficiency alone on cholinergic parameters have been investigated, the effect of the interaction between the two treatments still has many obscure points. Considering that alcoholics without clinically identifiable WKS pathology may have some degree of cell damage that has been caused by subclinical thiamine deficiency (Harper, 1983; Homewood et al., 1997; Lishman, 1981), we decided to assess the contribution of thiamine deficiency, associated or not with a chronic ethanol consumption in causing central cortical and hippocampal cholinergic alterations and open-field behavioral impairment. We used a mild thiamine deficiency experimental model in which animals were tested before the onset of neurological symptoms. Four categories of open-field behavior (sniffing, staring, grooming, and resting) were assessed and two biochemical functional parameters were analyzed: (i) activity of AChE in the cerebral cortex and hippocampus and (ii) release of ACh from slices of cerebral cortex in unstimulated and stimulated conditions. Histopathological evaluations were carried out in brain and liver tissues, using hematoxylin–eosin staining.

2. Materials and methods

2.1. Animals and treatments

Thirty-six male Wistar (200–250 g) 2-month-old rats were initially split into two groups, both treated for 4 months with tap water (W, $n=18$) or 20% (v/v) ethanol solution (E, $n=18$) as their only source of fluid. In the 12th week, the animals from each group were tested in the open-field (Session 1, described below). In the beginning of the 4th month, rats from the W and E groups were redivided into two groups each, resulting in four subgroups ($n=9$ in each group) in which animals were treated as follows: (i) WS: water and a standard control diet; (ii) WTD30: water and a thiamine-deficient diet in the last 30 days of the treatment; (iii) ES: ethanol solution and a standard control diet; and (iv) ETD7: which received ethanol associated with a thiamine-deficient diet only for the last 7 days of the treatment. The episode of thiamine deficiency (7 or 30 days) was interrupted before the animals started to show neurological symptoms. Chow and liquid consumption were recorded weekly and daily, respectively, and body weight was recorded every 15 days throughout treatment. In the last week of the 4th month, the animals were submitted to a second session of open-field tests. After that, all animals (from both chronic and acute experiments) were sacrificed for biochemical and morphological studies as described below. In order to be sure that the biochemical effects observed in rats of groups ETD30, ETD7, and ES were in fact induced by chronic ethanol treatment, and not due to a direct effect caused by the presence of ethanol in blood, we carried out a separate experiment in which eight rats were submitted to intraperitoneal injections of saline (control, $n=4$) or ethanol ($n=4$; single dose: 1.5 g/kg). Twenty minutes after the intraperitoneal injections, the animals were sacrificed for biochemical study as described below.

2.2. Open-field behavior test

The animals had their behavior observed in open-field tests in two sessions. Session 1 took place in the 12th week, before the onset of the thiamine deficiency episode, and Session 2 on the last day of thiamine deficiency treatment. Each rat was placed in the center of a white wooden box ($80 \times 80 \times 50$ cm) and observed for 5 min. Spontaneous open-field behavior was recorded every 20 s, using the momentary time–sample technique (Powell et al., 1975). The categories of behavior observed followed the criteria established by other authors (Barclay et al., 1981). Four categories were recorded: (i) sniffing, if the animal showed rapid movements of its nose and whiskers while moving or rearing with the forepaws in contact with the open-field wall; (ii) grooming, if it was biting the fur, scratching, or cleaning whiskers; (iii) resting, if it did not move and had a relaxed body posture; or (iv) staring, if it had a tense body posture, or stood on its hind legs, away from the wall, or if it rhythmically and slowly swayed from side to side (Barclay and Gibson, 1982).

2.3. Ethanol and thiamine blood levels

The method used to assess blood thiamine (Warnock, 1975) is based on the activity of the erythrocyte enzyme transketolase. Ethanol blood levels were determined by the Newman method (Moraes et al., 1991). Approximately 1500 μl of whole blood was collected, at the moment of the animal's sacrifice by decapitation, in an Eppendorf tube containing ethylenediaminetetraacetate as the anticoagulant. The samples were kept refrigerated at −40°C until the day of the analysis. Each sample was assayed twice using 50 and 500 μl of the whole blood for thiamine and ethanol determinations, respectively. Blood ethanol and thiamine levels were expressed as decigrams of ethanol per liter of blood and micromoles of ribose-5-phosphate consumed per hour per milliliter of blood, respectively.

2.4. Cholinergic parameters

The cholinergic parameters were determined in brain slices from every rat of each subgroup. AChE activity was measured separately in two brain areas, cortex and hippocampus, while ACh release was assessed only in the cortex. As mentioned, rats were killed by decapitation and the brain was rapidly removed and kept on ice. Cortex and hippocampus from one of the hemispheres was immediately dissected and sliced in prisms of 400 μm in a McIlwain tissue chopper. Approximately 30 mg of the homogeneous tissue from the whole cortex was transferred to tubes containing 1 ml of incubation medium for carrying out the ACh release study as described below. For the AChE activity, aliquots of approximately 20 mg of tissue from each area (whole cortex or hippocampus) were transferred to Eppendorf tubes containing 1 ml of borate buffer and frozen at −20°C until the day of the assay.

2.5. Acetylcholine release

Aliquots of 30 mg of cortical slices were preincubated under shaking for 5 min at 37°C in 1 ml of incubation medium containing (in mM): NaCl 136.0, KCl 2.7, $CaCl_2$ 1.35, $NaHCO_3$ 12.0, NaH_2PO_4 0.36, $MgCl_2$ 0.49, glucose 5.5, eserine 0.01. After 30 min of incubation in stimulated (potassium 30 mM) and unstimulated conditions, the reactions were stopped by adding 100 μl of TCA 50% to each tube. All tubes were kept at −20°C until the day of the assay, which was carried out in 2 days. The ACh released from the cortical slices was extracted (Prado et al., 1990) and measured (Israel and Lesbats, 1981) using the previously described methods (Prado et al., 1990). The ACh released was expressed as picomoles per milligram of tissue.

2.6. AChE activity assay

Tissue samples in 1 ml of 0.1 M borate buffer pH 8.2 were homogenized and assayed for AChE activity (Ellman et al., 1961). A volume of 135 μl of homogenate was added to a cuvette containing the following reagents: 35 μl of 5 mM dithiobisnitrobenzoic acid, 10 μl of 75 mM acetylthiocholine (ATCh), and 0.1 M borate buffer, pH 8.2, was added to make a final volume of 1 ml. The development of color was analyzed at 412 nm, using a recording spectrophotometer (UV-160[A] Shimadzu). The AChE activity was expressed in moles of ATCh hydrolyzed per minute per gram of tissue.

2.7. Morphological studies of the brain and liver tissues

Histopathological evaluations were made in liver slices and in the following brain areas: frontal, temporal, parietal and occipital lobes, basal ganglia, thalamus, mammillary bodies, midbrain, pons, medulla, and cerebellum. One hemisphere of each brain and fragments of liver tissue were fixed in 4% paraformaldehyde, processed to be embedded in paraffin, and cut in coronal sections 5 μm thick. Hematoxylin–eosin staining was used for histopathological evaluation under optical microscopy. The examiners were unaware about which animal group liver and brain samples came from.

2.8. Statistical analysis

Body weight data were analyzed using analysis of variance (ANOVA) with repeated measures. The mean biochemical parameter values and behavioral data for each group were compared through a one-way ANOVA. The post hoc test used to conduct multiple comparisons, in all cases, was the Bonferroni test. All tests were considered statistically significant at the 5% level.

3. Results

3.1. Acute ethanol experiment: AChE activity and ACh release

The mean ethanol blood level of the animals to whom ethanol intraperitoneal injections were administered was 4.5 ± 2.5 dg/l. AChE activities were not different in the two groups both in the cortex (control $= 8.9 \times 10^{-7} \pm 6.8 \times 10^{-8}$; ethanol $= 9.5 \times 10^{-7} \pm 8.7 \times 10^{-8}$) and in the hippocampus (control $= 1.2 \times 10^{-6} \pm 3.3 \times 10^{-8}$; ethanol $= 1.2 \times 10^{-6} \pm 6.6 \times 10^{-8}$). Similarly, the difference of cortical ACh release between animals from acute ethanol and control groups was not statistically different (unstimulated: control $= 5.9 \pm 0.6$, ethanol $= 6.0 \pm 0.5$; stimulated: control $= 22.9 \pm 1.0$, ethanol $= 21.7 \pm 1.1$).

3.2. Ethanol and thiamine blood levels of chronically treated animals

Fig. 1 shows that during the 16 weeks of treatment, animals from all groups gained weight ($P < .01$). Groups ETD7 (in the end of 2nd month) and WTD30 (in the beginning of 4th month) gained less weight compared to WS but these differences were not significant. Using Bonferroni tests for multiple comparisons in average weight of each experimental group, the following was observed: there were no significant differences between the groups, either at the beginning or at the end of the treatment. From the beginning of the 1st month until the end of 3rd month, the ETD7 group showed less body weight gain compared to the groups that did not take ethanol–WS and WTD30 ($P < .05$). Although the animals drinking ethanol solution ingested less chow compared to those from the water groups, the amount consumed was greater than 12–15 g/day, which is required for an adult rat (Warner, 1962). We observed a great individual variance in the ethanol blood levels. The values ranged from 1.8 to 17.0 dg/l. The mean value found was of 6.9 ± 3.2 dg/l. Average blood thiamine levels in micromoles of ribose-5-phosphate consumed per hour per milliliter of blood were: WTD30 $= 5.67 \pm 0.24$ and ETD7 $= 5.48 \pm 0.13$. These values are significantly lower ($P < .05$) when compared to WS (22.27 ± 0.91) and ES (20.05 ± 2.09).

3.3. Behavioral studies

A one-way ANOVA was performed for each of the two sessions to compare the four groups WS, WTD30, ES, and ETD7. There were no significant differences between groups concerning open-field behaviors assessed in Session 1 (data not shown). Fig. 2 shows the results of open-field behavior in Session 2. The vertical axis of each graph represents the mean number of times the behavior was recorded, as shown in Panels A, B, C, and D for behavioral categories "staring," "grooming," "resting," and "sniffing." For the first three categories — "staring," "grooming," and "resting" — there was no difference among the

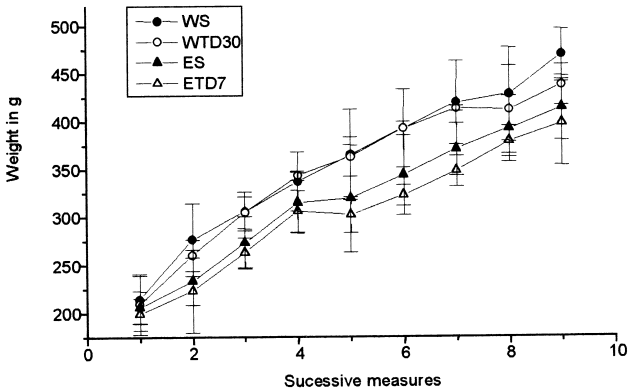

Fig. 1. Mean ± S.D. weight measured every 2 weeks for groups WS ($n = 9$), WTD30 ($n = 9$), ES ($n = 9$), and ETD7 ($n = 9$).

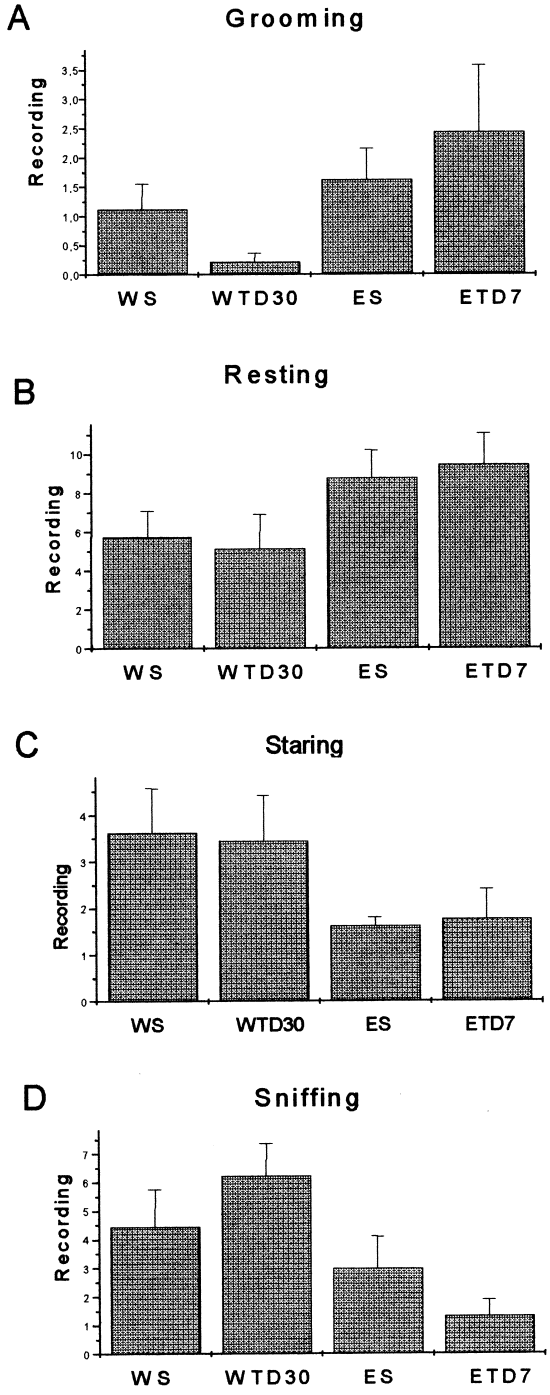

Fig. 2. Mean ± S.E. of frequency of four behavioral categories: sniffing, staring, grooming, and resting (Panels A, B, C, and D, respectively) for groups WS ($n=9$), WTD30 ($n=9$), ES ($n=9$), and ETD7 ($n=9$). The only significant difference is in the "sniffing category," between groups WTD30 and ETD7.

groups. In the "sniffing" category, the ANOVA shows a significant difference among the four groups ($P=.020$). And the post hoc test of Bonferroni indicates that there is a significant difference between groups WTD30 and ETD7 ($P=.017$), with the former presenting a higher response frequency than the latter.

3.4. AChE activity assay

The data obtained from AChE activity assay, in cerebral cortex and hippocampus, are shown in Fig. 3. In cerebral cortex and hippocampus, one-way ANOVA showed a significant ($P<.05$) difference among the four groups. In the cortex, the multiple comparisons, using the post hoc Bonferroni test, indicated that groups WS and ES present higher AChE activities ($P<.001$) compared to groups with thiamine deficiency (WTD30 and ETD7). In the hippocampus, post hoc tests showed that group WS had a higher enzyme activity than group ES ($P<.05$). Furthermore, WTD30 and ETD7 groups did not differ significantly from each other, but showed significantly lower activity compared to ES ($P<.01$). Therefore, these data show that in cerebral cortex, AChE activity was reduced by approximately 44% and 45% in animals from WTD30 and ETD7 groups, respectively, while no reduction was found in animals from ES group. In hippocampus, there was a more pronounced (62%, 64%, and 33%) reduction in animals from WTD30, ETD7, and ES groups, respectively.

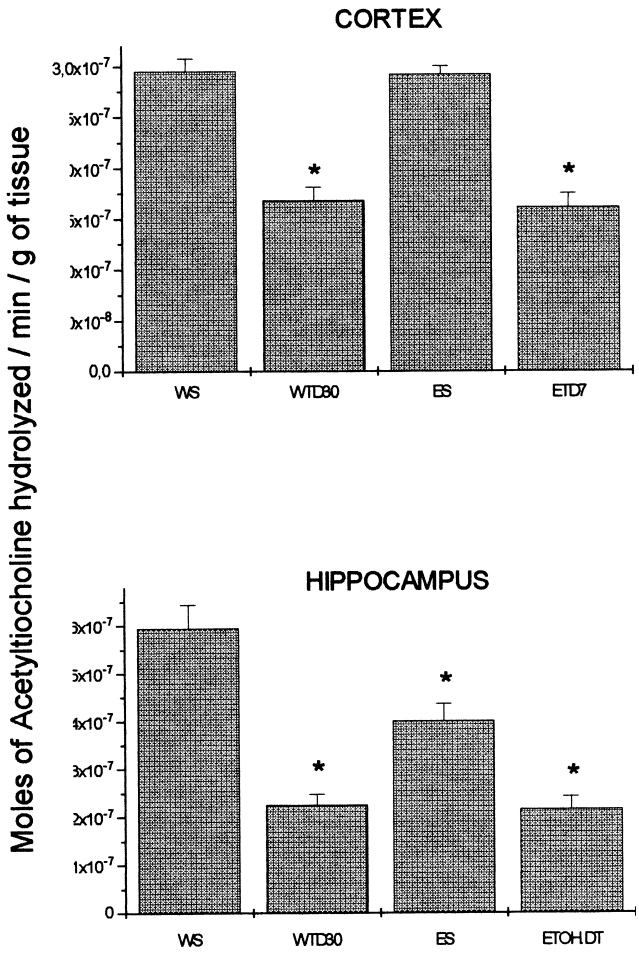

Fig. 3. Mean ± S.E. AChE activity (moles of acetylthiocholine hydrolyzed per minute per gram of tissue) in the cortex and hippocampus for groups WS ($n=9$), WTD30 ($n=9$), ES ($n=8$), and ETD7 ($n=9$). Different characters above bars mean significant differences ($P<.05$).

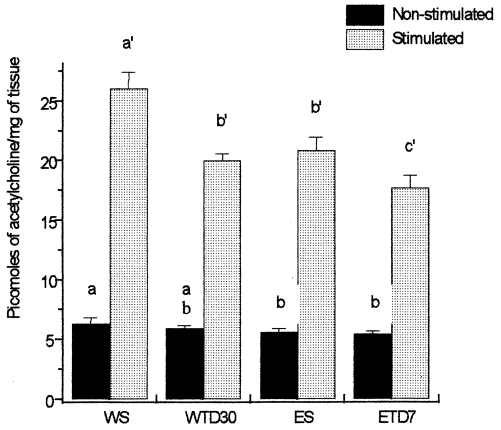

Fig. 4. Mean ± S.E. ACh release (picomoles per milligram of tissue in 30 min) in stimulated and nonstimulated conditions for groups WS ($n = 8$), WTD30 ($n = 8$), ES ($n = 8$), and ETD7 ($n = 8$). Different characters above bars mean significant differences ($P < .05$).

3.5. Acetylcholine release

The mean values of released ACh in cerebral cortex slices for the four groups are shown in Fig. 4. Under stimulated and nonstimulated conditions, the F test has indicated significant differences among the four groups ($P < .05$). Bonferroni-paired comparisons showed that, under nonstimulated conditions, group WS presented higher ACh release than groups ES and ETD7 ($P < .005$). ACh release in the WTD30 group was not different from WS, ES, and ETD7 groups ($P > .05$). Under stimulated conditions, a significant decrease of ACh release was found for all experimental groups compared to WS ($P < .001$). There were no significant differences between groups WTD30 and ES, but both showed higher ACh release compared to ETD7 group ($P < .01$).

3.6. Morphological studies

Assessed by optical microscopy, histological sections of brain and liver tissue from the animals of experimental and control groups showed no detectable morphological alterations. All cerebral regions examined, where usually lesions due to thiamine deficiency treatment are described in the literature, showed normal structural aspects (data not shown).

4. Discussion

Animals treated with ethanol gained less weight and had a lower average body weight, but there were no significant differences between the groups either at the beginning or at the end of the treatment. This finding is in accordance with other authors (Lieber and DeCarli, 1994) who have shown that ethanol consumption induces a decrease in food ingestion. However, the animals of ES and ETD7 groups were not undernourished and this is supported by the following observations: they ingested more chow than the amount required per day, and liver histopathological evaluation showed no morphological change. Besides, ethanol-treated animals did not lose weight during the 4 months of treatment. Even though WTD30 group chow consumption decreased in the last 3 weeks of the treatment ($P < .05$), it did not result in significant loss of body weight.

The animals from both ethanol-treated groups, ES and ETD7, showed large individual variability in ethanol consumption and this was reflected in their ethanol blood levels, which ranged from 1.8 to 17.0 dg/l with a mean value of 6.9 dg/l. These data are in accordance with other authors who found values ranging from 2.4 to 16.7 dg/l (Hodges et al., 1991; Sousa et al., 1995; Tavares et al., 1987). These variations may be due both to individual differences between the animals and to the different amounts of ethanol solution ingested prior to blood sample collection.

Although all animals on the thiamine-deficient diet showed significantly low blood levels of thiamine, we did not observe clinical symptoms of this vitamin deficiency in any animal, except those from WTD30 group, which started to show one of the first clinical symptoms, anorexia, in the beginning of the 3rd week of treatment. However, the animals from this group showed no signs of neurological alteration. Hence, in the present work, the data obtained refer to the subclinical initial stage of the thiamine deficiency pathological process. Thiamine blood levels of animals from groups WTD30 and ETD7 were almost the same, despite the difference in the duration of the episode of thiamine deficiency between these groups. Several factors may contribute to the acceleration of the process of thiamine deficiency in ethanol chronically treated rats, including impairment of thiamine tissue storage (Abe and Itokawa, 1977) and impairment of intestinal absorption (Hoyumpa, 1980; Thomson et al., 1968; Tomasulo et al., 1968).

Histopathological evaluation by optical microscopy of thiamine-deficient animals showed no changes in the examined brain areas. Several studies using animal models with different durations of thiamine deficiency episodes have shown lesions in different regions of the brain (Kril and Homewood, 1993; Langlais and Savage, 1995). However, we have to consider that in the present work the episode of thiamine deficiency was interrupted before the animals started to show neurological symptoms. Literature data show that animals treated with a thiamine-deficient diet show anorexia and weight loss after 2–3 weeks and intermittent seizures with opisthotonos after 4–8 weeks on the diet (Plaitakis et al., 1982). Levels of thiamine had been quantified in different organs and tissues in animals submitted to thiamine deficiency for 4 weeks. In this work, the authors demonstrate that the decrease in thiamine level of the brain was seen only after 3 weeks of treatment and that this decrease is slower compared to other organs (Molina et al., 1994).

Behavioral results showed that only in the "sniffing" category were there significant differences among the four experimental groups. Inspection of Fig. 2 shows that the association of chronic ethanol and 7-day thiamine deficiency treatment changes this behavior in an opposite direction when compared to 30-day thiamine deficiency treatment alone. No other behavioral category showed significant differences caused by either treatment. Sniffing comprises the joint recording of ambulation and rearing, being, unlike the other categories (grooming, staring, and resting), an exploratory category of behavior. So, our data suggest that thiamine deficiency causes some specific effect on exploratory behavior. Regarding staring behavior, unlike other authors (Barclay and Gibson, 1982), we did not observe any increase in the frequency of this behavior caused by thiamine deficiency. This is possibly due to differences in the recording techniques and/or the thiamine deficiency model used. They decided which category of behavior was predominant in each 6-s interval (Barclay and Gibson, 1982), while we used the momentary time sampling procedure. Thus, in our work, the observer glanced at the animal every 20 s and recorded the behavior displayed at the exact moment of the glance. The method used by us was possibly less sensitive than that used by Barclay and Gibson, because we used a larger interobservation interval. However, it is less subjective, because the observer does not have to decide, as they did, on the predominant behavior exhibited during the interval. It has been shown that momentary time sampling with a 20-s interobservation interval can produce data with greater correspondence to the "true" total duration of the activity (Powell et al., 1975). Furthermore, unlike Barclay and Gibson's (1982) data, ours were gathered by observers that were unaware of which group the animal came from. In addition, contrary to Barclay and Gibson, we did not use pyrithiamine associated with a thiamine-deficient diet to induce thiamine deficiency.

In the cerebral cortex, AChE activity decreased significantly in animals from the WTD30 and ETD7 groups. Animals from ES group did not show any change in the activity of this enzyme. A previous study carried out by our group showed that after 8 months of ethanol intake and 2 months of abstinence, cortical AChE activity was unchanged (Pereira et al., 1998). However, in hippocampus, as we can see in Fig. 3, both ethanol (ES) and thiamine deficiency (WTD30) treatments have a significant decreasing effect on AChE activity. Seven days of thiamine deficiency treatment associated with ethanol has the same effect as a longer (30 days) thiamine deficiency treatment alone.

Regarding ACh release studies, under a nonstimulated condition, ethanol treatment, associated or not with 7-day thiamine deficiency decreased ACh release, while 30-day thiamine deficiency alone had no effect. Under a stimulated condition, reductions were found for groups WTD30, ES, and mainly ETD7, whose reduction was even greater. These data suggest that both thiamine deficiency for 30 days and chronic ethanol treatment induce reduction in tissue response for ACh release, but the association of these two treatments for only 7 days induces a greater reduction than the effect caused by each of them separately.

The biochemical alterations found here were caused by chronic intake of ethanol and not by a direct acute effect. This conclusion is supported by the acute experiment, where the animals received only one intraperitoneal ethanol dose that did not affect cholinergic parameters although their ethanol blood levels were similar to the chronic ethanol-treated animals. Literature data are in accordance with our results. Experiments on the in vitro ethanol effect on cholinergic parameters have shown that ethanol has no effect on spontaneous ACh release and did not interfere with the ACh response to high potassium stimulation (Sunahara and Kalant, 1980).

In this work, experimental animals showed behavioral and biochemical changes, however, no correlation between sniffing and the biochemical data both in hippocampus and cortex was found (results not shown). No morphological alterations were observed. These data indicate that the pathological process studied was in its initial stage. It is already known that chronic ethanol and thiamine deficiency can induce biochemical dysfunction that precedes the morphological lesion usually found after an episode of the deficiency (Gibson et al., 1989; Pratt et al., 1990).

Another experiment (results not shown) with a small group of rats ($n = 5$) in which we studied the effect of 7 days of thiamine deficiency diet treatment alone showed that blood level of thiamine were lower compared to control animals (WS and ES groups), and were greater than the average level of ETD7 group for which thiamine deficiency episode duration was the same. It suggests that ethanol intake interferes in the level of thiamine in blood, which is in accordance to previous observation that ethanol affects thiamine absorption through the gastrointestinal tract (Hoyumpa, 1980; Thomson et al., 1968; Tomasulo et al., 1968). There was no change in the activity of AChE in the hippocampus of animals in the WTD7 group. These preliminary data suggest that thiamine deficiency for 7 days did not cause changes in cortical AChE activity unless it was associated with ethanol intake.

In short, the present results show that thiamine deficiency for 30 days reduces AChE activity in both cortex and hippocampus. Four months of ethanol consumption did not change cortical AChE activity while 7 days of ethanol intake associated with a thiamine-deficient diet significantly reduced the enzyme activity to the same extent as did 30 days of thiamine deficiency alone.

Our results suggest that the cholinergic system seems to respond differently to thiamine deficiency and to chronic ethanol consumption; and the association of both treatments seems to induce a higher effect on ACh release stimulated by high potassium concentration than that caused by each of them alone. Our data also suggest that the effect of chronic ethanol consumption and thiamine

deficiency on sniffing behavior is not synergistic. The data obtained from the present work indicate that in the initial stages of thiamine deficiency brain dysfunction, before the development of either WKS brain structural lesions or neurological symptoms, chronic ethanol consumption can modify the biochemical dysfunctions induced by this thiamine deficiency.

Acknowledgments

This work was supported by FAPEMIG (Fundação de Amparo à Pesquisa de Minas Gerais) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). Rita G.W. Pires received a scholarship from FAPEMIG.

References

Abe T, Itokawa Y. Effect of ethanol administration on thiamine absorption and transketolase activity in rats. Int J Vitam Nutr Res 1977;47:307–14.

Arendt T, Allen Y, Sinden J, Schugens M, Marchbanks RM, Lantos PL, Gray JÁ. Cholinergic-rich brain transplants reverse alcohol-induced memory deficits. Nature 1988a;332:448–50.

Arendt T, Hennig D, Gray JÁ, Marchbanks R. Loss of neuron in the rat basal forebrain cholinergic projection system after prolonged intake of ethanol. Brain Res Bull 1988b;21:563–70.

Arendt T, Allen Y, Marchbanks RM, Schugens MM, Sinden J, Lantos PL, Gray JÁ. Cholinergic system and memory in the rat: effects of chronic ethanol, embryonic basal forebrain transplants and excitotoxic lesions of cholinergic basal forebrain projection system. Neuroscience 1989;33: 435–62.

Bâ A, Seri BV, Aka KJ, Glin L, Tako A. Comparative effects of developmental thiamine deficiencies and ethanol exposure on the morphometry of the CA3 pyramidal cells. Neurotoxicol Teratol 1999;21:579–86.

Barclay LL, Gibson GE. Spontaneous open-field behavior in thiamin-deficient rats. J Nutr 1982;112:1899–905.

Barclay LL, Gibson GE, Blass JP. Impairment of behavior and acetylcholine metabolism in thiamine deficiency. J Pharmacol Exp Ther 1981; 217:537–43.

Barclay LL, Gibson GE, Blass JP. Cholinergic therapy of abnormal open-field behavior in thiamine-deficient rats. J Nutr 1982;112:1906–13.

Blokland A, Prickaerts J, Raaijmakers W. Absence of impairments in spatial and temporal discrimination learning in Lewis rats after chronic ethanol consumption. Pharmacol, Biochem Behav 1993;46:27–34.

Cadete-Leite A, Andrade JP, Sousa N, Ma W, Ribeiro-da-Silva A. Effects of chronic alcohol consumption on the cholinergic innervation of the rat hippocampal formation as revealed by choline acetyltransferase immunocytochemistry. Neuroscience 1995;64:357–74.

Casamenti F, Scali C, Vannucchi MG, Bartolini L, Pepeu G. Long-term ethanol consumption by rats: effect on acetylcholine release in vivo, choline acetyltransferase activity, and behavior. Neuroscience 1993;56: 465–71.

Ciccia RM, Langlais PJ. An examination of the synergistic interaction of ethanol and thiamine deficiency in the development of neurological signs and long-term cognitive and memory impairments. Alcohol: Clin Exp Res 2000;24:622–34.

Ellman GL, Courtney KD, Andres VJ, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

Floyd EA, Young-Seigler AC, Ford BB, Reasor JD, Moore EL, Townsel HK, Rucker HK. Chronic ethanol ingestion produces cholinergic hypofunction in rat brain. Alcohol 1997;14:93–8.

Gibson G, Barclay L, Blass J. The role of the cholinergic system in thiamine deficiency. Ann NY Acad Sci 1982;378:382–403.

Gibson GE, Nielsen P, Mykytyn V. Regionally selective alterations in enzymatic activities and metabolic fluxes during thiamine deficiency. Neurochem Res 1989;14:17–24.

Harper CG. The incidence of Wernicke's encephalopathy in Australia. A neuropathological study of 131 cases. J Neurol Neurosurg Psychiatry 1983;46:593–8.

Hodges H, Allen Y, Sinden J, Mitchell SN, Arendt T, Lantos PL, Gray JÁ. The effects of cholinergic drugs and cholinergic-rich foetal neural transplants on alcohol-induced deficits in radial maze performance in rats. Behav Brain Res 1991;43:7–28.

Homewood J, Bond NW, Mackenzie A. The effects of single and repeated episodes of thiamine deficiency on memory in alcohol-consuming rats. Alcohol 1997;14:81–91.

Hoyumpa AM. Mechanisms of thiamine deficiency in chronic alcoholism. Am J Clin Nutr 1980;33:2750–61.

Israel M, Lesbats B. Continuous determination of transmitter release and compartmentation in *Torpedo* electric organ synaptosomes, studied with chemiluminescent method detecting acetylcholine. J Neurochem 1981; 37:1474–83.

Kril JJ. The contribution of alcohol, thiamine deficiency and cirrhosis of the liver to cerebral cortical damage in alcoholics. Metab Brain Dis 1995; 10:9–16.

Kril JJ, Homewood J. Neuronal changes in the cerebral cortex of the rat following alcohol treatment and thiamine deficiency. J Neuropathol Exp Neurol 1993;52:586–93.

Langlais PJ, Savage LM. Thiamine deficiency in rats produce cognitive and memory deficits on spatial tasks that correlate with tissue loss in diencephalon, cortex and white matter. Behav Brain Res 1995;68: 75–89.

Lieber CS, DeCarli LM. Animal models of chronic ethanol toxicity. Methods Enzymol 1994;233:585–94.

Lishman WA. Cerebral disorders in alcoholism. Syndromes of impairments. Brain 1981;104:1–20.

Martin PR, McCool BA, Singleton CK. Genetic sensitivity to thiamine deficiency and development of alcoholic organic brain disease. Alcohol: Clin Exp Res 1993;17:31–7.

Mesulam MM, Mufson EJ, Wainer BH, Levey AL. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). Neuroscience 1983;10:1185–201.

Molina PE, Myers N, Smith RM, Lang CH, Yousef KA, Tepper PG, Abumrad N. Nutritional and metabolic characterization of a thiamine-deficient rat model. J Parenter Enteral Nut 1994;18:104–12.

Moraes ECF, Fzmelwav RB, Fernicola NAGGD. Manual de toxicologia analítica 2nd ed. São Paulo: Ed. Rocca, 1991.

Ogishima M, Hiraga Y, Washizuka M, Kobayashi N, Nakata N, Ikeda Y. Differential effects of intracerebroventricular AF64A injection, nucleus basalis of Meynert lesion, and scopolamine on place navigation and open-field behavior of rats. Yakubutsu, Seishin, Kodo 1992;12:285–92.

Palencia G, Teixeira F, Ortiz A, Perez R, Rios C, Sotelo J. Detrimental effects of malnutrition on the damage induced by alcoholism: a study of animal models the simulate chronic alcoholism and malnutrition of large human groups. J Stud Alcohol 1994;55:113–20.

Pelham RW, Marquis JK, Kugelmann K, Munsat TL. Prolonged ethanol consumption produces persistent alterations of cholinergic function in rat brain. Alcohol: Clin Exp Res 1980;4:282–7.

Pereira SRC, Menezes GA, Franco GC, Costa AEB, Ribeiro AM. Chronic ethanol consumption impairs spatial remote memory in rats but does not affect cortical cholinergic parameters. Pharmacol, Biochem Behav 1998;60:305–11.

Pittella JEH, Giannetti AV. Morphometric study of the neurons in the medial mammary nucleus in acute and chronic Wernicke's encephalopathy. Clin Neuropathol 1994;13:26–31.

Plaitakis A, Hwang EC, Van Woert MH, Szilagyi PIA, Berl S. Effect of thiamin deficiency on brain neurotransmitter systems. Ann NY Acad Sci 1982;378:367–81.

Powell J, Martindale A, Kulp S. An evaluation of time–sample measures of behavior. J Appl Behav Anal 1975;8:463–9.

Prado MA, Moraes-Santos T, Freitas RN, Silva MA, Gomez MV. Choline oxidase chemiluminescent assay, after removal of eserine from medium, of acetylcholine released in vitro from brain slices. J Neurosci Methods 1990;31:193–6.

Pratt OE, Rooprai HK, Shaw GK, Thomson AD. The genesis of alcoholic brain tissue injury. Alcohol Alcohol 1990;25:217–30.

Rao VL, Mousseau DD, Butterworth RF. A quantitative autoradiographic study of muscarinic cholinergic receptor subtypes in the brains of pyrithiamine-treated rats. Neurochem Res 1995;20:907–14.

Rawat AK. Brain levels and turnover rates of presumptive neurotransmitter as influenced by administration and withdrawal of ethanol in mice. J Neurochem 1974;22:915–22.

Ron MA, Acker W, Shaw GK, Lishman WA. Computerized tomography of the brain in chronic alcoholics: a survey and follow-up study. Brain 1982;105:497–514.

Smyth RD, Beck H. The effect of time and concentration of ethanol administration on brain acetylcholine metabolism. Arch Int Pharmacodyn Ther 1969;182:295–9.

Sousa N, Madeira MD, Ruela C, Paula-Barbosa MM. Structural reorganization in the supraoptic nucleus of withdrawn rats following long-term alcohol consumption. Alcohol: Clin Exp Res 1995;19:879–85.

Sunahara GI, Kalant H. Effect of ethanol on potassium-stimulated and electrically stimulated acetylcholine release in vitro from rat cortical slices. Can J Physiol Pharmacol 1980;58:706–11.

Tavares MA, Paula-Barbosa MM, Cadete-Leite A. Chronic alcohol consumption reduces the cortical layer volumes and the number of neurons of the rat cerebellar cortex. Alcoholism 1987;11:315–9.

Thomson A, Baker H, Leevy CM. Thiamine absorption in alcoholism. Am J Clin Nutr 1968;21:537–8.

Tomasulo PA, Kater RMH, Iber FL. Impairment of thiamine absorption in alcoholism. Am J Clin Nutr 1968;21:1341–4.

Torres EM, Perry TA, Blockland A, Wilkinson LS, Wiley RG, Lappi DA, Dunnet SB. Behavioural, histochemical and biochemical consequences of selective immunolesions in discrete regions of the basal forebrain cholinergic system. Neuroscience 1994;63:95–122.

Warner RG. Nutrient requirements of the laboratory rat. Nutrient requirements of laboratory animals. National Academy of Sciences–National Research Council. Washington (DC): National Academy of Sciences, 1962. pp. 51–95 (Publication 990).

Warnock LG. Transketolase activity of blood hemolysate, a useful index for diagnosing thiamine deficiency. Clin Chem 1975;21:432–6.