Neuroscience 135 (2005) 1129–1139

THIAMINE DEFICIENCY IN THE PATHOGENESIS OF CHRONIC ETHANOL-ASSOCIATED CEREBELLAR DAMAGE *IN VITRO*

P. J. MULHOLLAND,* R. L. SELF,[a] T. D. STEPANYAN,[b] H. J. LITTLE,[c] J. M. LITTLETON[d] AND M. A. PRENDERGAST[a]

[a]*Department of Psychology, 012-I Kastle Hall, University of Kentucky, Lexington, KY 40506-0044, USA*

[b]*Department of Molecular and Biomedical Pharmacology, University of Kentucky College of Medicine, A.B. Chandler Medical Center MS 305, Lexington, KY 40536-0298, USA*

[c]*Departments of Addictive Behavior and Pharmacology, St George's Hospital Medical School, SW17 ORE, London, UK*

[d]*Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, USA*

Abstract—Nutritional deficiencies associated with long-term ethanol consumption may cause neuronal damage in ethanol-dependent individuals. Thiamine deficiency, in particular, is thought to contribute to ethanol-associated cerebellar degeneration, although damage may occur in adequately nourished alcoholics. Thus, the present study examined the effects of thiamine depletion and ethanol exposure on cytotoxicity in rat cerebellum. Organotypic cerebellar slice cultures were treated starting at 25 days *in vitro* with 100 mM ethanol for 11 days or 10 days followed by a 24-h withdrawal period. This exposure paradigm has previously been shown in hippocampal slice cultures to result in spontaneous cytotoxicity upon ethanol withdrawal. Additional cerebellar cultures were exposed to the thiamine depleting agent pyrithiamine (10–500 μM) for 10 or 11 days, some in the presence of ethanol exposure or withdrawal. Other cultures were co-exposed to thiamine (1–100 μM), 500 μM pyrithiamine, and ethanol for 10 or 11 days. The results demonstrated that neither 11-day ethanol treatment nor withdrawal from 10-day exposure significantly increased cerebellar cytotoxicity, as measured by propidium iodide fluorescence. The 11-day treatment with 100 or 500 μM pyrithiamine significantly increased propidium iodide fluorescence ~21% above levels observed in control tissue. Cultures treated with both ethanol (11 days or 10 days plus withdrawal) and 500 μM pyrithiamine displayed a marked increase in cytotoxicity ~60–90% above levels observed in control cultures. Pyrithiamine and ethanol-induced cytotoxicity was prevented in cultures co-exposed to thiamine (10–100 μM) for the duration of pyrithiamine treatment. Findings from this report suggest that the cerebellum may be more sensitive to the toxic effects of thiamine deficiency, as compared with alcohol withdrawal, associated with alcohol dependence. © 2005 Published by Elsevier Ltd on behalf of IBRO.

*Correspondence to: P. J. Mulholland, Medical University of South Carolina, Center for Drug and Alcohol Programs, 67 President Street, Charleston, South Carolina 29425, USA. Tel: +1-843-792-5159; fax: +1-843-792-7353.
E-mail address: mulholl@musc.edu (P. J. Mulholland).

Abbreviations: DIV, days *in vitro*; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HIHS, heat-inactivated horse serum; NMDA, *N*-methyl-D-aspartate.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.06.077

Key words: alcoholism, malnutrition, ethanol withdrawal, pyrithiamine, neuronal damage.

Rates of alcohol (ethanol) abuse and dependence, prevalent worldwide disorders (World Health Organization, 2001), have increased in the United States from 7.41% in 1991–1992 to 8.46% in 2001–2002, affecting nearly 18 million adults (Grant et al., 2004). Ethanol-related disorders not only reduce the quality of life of those afflicted with these disorders, but also affect society in general. Lifetime heavy ethanol consumption is associated with the development of neurological abnormalities and reduced volume of cortical and subcortical structures (Hommer et al., 2001; Mann et al., 2001), including the cerebellum (Andersen, 2004). Many of the neurological deficits, which appear in 50–75% of long-term ethanol-dependent individuals (Eckardt and Martin, 1986), are thought to result from nutritional deficiencies (Charness, 1993; Diamond and Messing, 1994; Fadda and Rossetti, 1998; Harper and Kril, 1990), excessive consumption over many years (Jacobson and Lishman, 1990), and repeated withdrawal from chronic ethanol intake (Duka et al., 2004; Hunt, 1993). In addition, these deficits may result from secondary effects, such as liver dysfunction (Tarter et al., 1986) or hypothalamic–pituitary–adrenal axis alterations (Adinoff et al., 1998; Mulholland et al., 2005; van Thiel and Lester, 1978).

Some alcoholics may suffer from malnutrition, particularly demonstrating deficiencies in vitamins A, B_1 (thiamine), B_2 (riboflavin), and C (ascorbic acid), folic acid, magnesium, carbohydrates, fat, and protein, likely due to reduced nutritional intake or decreased absorption from an adequate diet (for review, see Lieber, 2003). Many studies have focused on the role of thiamine in ethanol-related toxicity given that thiamine deficiency may cause brain damage in non-alcoholic patients (Adeyinka et al., 1996; Kohn et al., 1997; Reuler et al., 1985; Victor et al., 1989). Thiamine is an essential nutrient and plays an important role in metabolic and cellular function, particularly within the brain. Thiamine and thiamine-utilizing enzymes are involved in brain excitability (Schoffeniels, 1990), in addition to carbohydrate and energy metabolism (Collins et al., 1970). Additionally, thiamine may be involved in synthesis of neurotransmitters, nucleic acids, fatty acids, and steroids (Martin et al., 2003).

Many alcoholics consume less than 0.29 mg/1000 kcal of thiamine in their diet compared with 0.4–2.0 mg/1000 kcal of thiamine in the diet of healthy individuals (Woodhill and Nobile, 1972). Ethanol exposure may impair the absorption of thiamine (Gastaldi et al., 1989; Hoyumpa, 1980) and reduce the activity of enzymes responsible for

thiamine utilization and metabolism (Laforenza et al., 1990; Lavoie and Butterworth, 1995; Poupon et al., 1990; Tallaksen et al., 1992), resulting in decreased brain thiamine levels even in the presence of adequate nutrition. Since long-term ethanol consumption and malnutrition in the absence of alcoholism are the most common pathological conditions that detrimentally affect function and viability of the cerebellum (Adeyinka et al., 1996; Kohn et al., 1997; Reuler et al., 1985; Victor et al., 1989), some posit that thiamine deficiency in alcoholics may be the cause of cerebellar damage (Charness, 1993; Diamond and Messing, 1994; Fadda and Rossetti, 1998; Harper and Kril, 1990).

Recent studies demonstrated that cerebellar Purkinje cell shrinkage and degeneration in ethanol-dependent individuals related to ataxia of the lower limbs (Andersen, 2004; Sullivan et al., 2000). Postmortem studies revealed that 20–50% of severe alcoholics show predominant atrophy of the superior vermis of the cerebellum (Karhunen et al., 1994; Shear et al., 1996; Torvik and Torp, 1986). Interestingly, the incidence of cerebellar damage is more marked in patients suffering from Wernicke's encephalopathy (Baker et al., 1999; Nicolas et al., 2000; Phillips et al., 1987), a disorder characterized by thiamine deficiency, confusion, ataxia, incoordination of the lower limbs, and oculomotor abnormalities. However, cerebellar atrophy may occur in alcoholics without ataxia (Torvik and Torp, 1986; Victor et al., 1989) or malnourishment (Nicolas et al., 2000). Thiamine deficiency in animal models resulted in cerebellar damage and memory impairments similar to those observed in Wernicke's patients (Blank et al., 1975; Langlais and Savage, 1995). Thus, it is unclear if cerebellar atrophy observed in alcoholics is the result of nutritional deficiencies, long-term ethanol consumption, repeated withdrawals, hypothalamic–pituitary–adrenal axis alterations, and/or liver disease.

Data from this laboratory previously reported spontaneous cytotoxicity upon withdrawal from 10-day ethanol exposure in organotypic hippocampal slice cultures (Harris et al., 2003; Mulholland et al., 2003; Prendergast et al., 2004). Using this model, the present set of studies attempted to determine the role of thiamine deficiency in cerebellar damage associated with chronic ethanol exposure and withdrawal. To this end, cerebellar slice cultures from neonatal rats were exposed to a 10-day ethanol treatment followed by a 24-h withdrawal period. Pyrithiamine, a selective antagonist of brain thiamine pyrophosphokinase and competitive inhibitor of the thiamine transporter (Casirola et al., 1988, 1990; Iwashima et al., 1975; Yoshioka, 1984), represents a vehicle to induce thiamine deficiency in animal models (Butterworth and Heroux, 1989; Gibson et al., 1989). Thus, additional cultures were exposed to pyrithiamine during ethanol treatment and withdrawal to assess a potential deleterious effect of thiamine deficiency in conjunction with chronic ethanol exposure and withdrawal. Cultures were also co-exposed to thiamine to determine if this would prevent the cytotoxicity associated with pyrithiamine and ethanol treatment.

EXPERIMENTAL PROCEDURES

Organotypic cerebellar slice culture preparation

Preparation of cerebellar cultures followed procedures described by Stoppini et al. (1991) with modifications as detailed below. Cerebellum from 8-day old male and female Sprague–Dawley rat pups (Harlan, Indianapolis, IN, USA) were aseptically removed and placed into cold (4 °C) dissecting medium (Minimum Essential Medium with 2 mM L-glutamine plus 25 mM HEPES and 50 μM penicillin/streptomycin solutions). Using a McIllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK), each cerebellum was sagittally sectioned at 350 μm and placed into fresh culture medium. Culture medium was composed of dissecting medium with the addition of 36 mM glucose, 25% (v/v) Hanks' balanced salt solution (HBSS), and 25% heat-inactivated horse serum (HIHS). Each cerebellum yielded ~16 slices. Two slices were randomly transferred onto individual Millicell-CM 0.4 μm biopore membrane inserts (Millipore, Marlborough, MA, USA) in 35 mm six-well culture plates containing 1 ml of pre-incubated culture medium. Excess medium on top of slices was aspirated to ensure cultures remained exposed to the atmosphere of 5% CO_2/95% air. Cultures were kept at 37 °C in an incubator at 95% humidity and were allowed to become attached to membrane inserts for 25 days prior to the start of the experiments. Culture medium was replenished every 5 days. Gibco BRL (Gaithersburg, MD, USA) supplied all culture medium solutions with the exception of HIHS (Sigma-Aldrich, Co., St. Louis, MO, USA). Thiamine concentration in culture medium was estimated to be ~2 μM. Given that the thiamine concentration of HIHS is unknown, the estimate for horse serum was based on thiamine concentrations from calf and goat serum. Care of all animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) in an effort to minimize the number of animals used and their suffering. All experiments were conducted in accordance with the University of Kentucky Institutional Animal Care and Use Committee guidelines.

Ethanol treatment and withdrawal

The method for ethanol treatment followed those employed in a previous study that demonstrated hippocampal toxicity due to ethanol withdrawal (Harris et al., 2003). At 25 days *in vitro* (DIV), all cultures (n=12/group) were randomly transferred to six-well culture plates containing 1 ml of standard culture medium, some with the addition of 100 mM ethanol. All six-well culture plates were placed in topless polyethylene chambers that contained either 50 ml distilled H_2O for untreated controls or 100 mM ethanol in 50 ml distilled H_2O for ethanol-treated groups. Chambers were then placed in sealable plastic bags. The bags were filled with medical grade compressed gas (5% CO_2, 21% O_2, and 74% N_2), sealed, and returned to the incubator. After five continuous days in the incubator, all cultures were removed from their respective treatment plates and placed in new plates containing medium with the identical treatment as the previous 5 days. All treatments and water baths were refreshed at this time to compensate for ethanol evaporation during the first five days of treatment. Measurement of ethanol in culture medium, as described in Prendergast et al. (2004), indicated that an actual ethanol concentration of 100 mM was ~91.6 mM and, after 5DIV, declined to ~42.6 mM. Thus, 100 mM ethanol is indicative of a calculated starting concentration. After 10 days of continuous ethanol exposure, withdrawal was induced in some cultures by replacing ethanol-containing medium with fresh culture medium lacking ethanol. Additional

cultures, including some that were not previously exposed to ethanol, were treated with 100 mM ethanol for 24-h starting at 35DIV. All slices were then placed in the incubator for 24-h prior to assessing ethanol-associated cytotoxicity by the addition of 2.5 μg/ml of propidium iodide (Molecular Probes™, Invitrogen Corporation, Eugene, OR, USA) in their respective treatment media. Previous research from this laboratory demonstrated in organotypic hippocampal slice cultures that withdrawal from 10-day exposure to 100 mM ethanol, but not 11-day continuous ethanol exposure, significantly increased propidium iodide fluorescence (Harris et al., 2003; Mulholland et al., 2003; Prendergast et al., 2004).

Pyrithiamine and thiamine exposure

To assess the effects of thiamine deficiency, cultures were exposed to pyrithiamine (hydrobromide; Sigma-Aldrich, Co.; 10–500 μM) for either 10 or 11 days starting at 25DIV. Additional cultures were co-exposed to pyrithiamine for either 10 or 11 days in the presence of 11-day 100 mM ethanol treatment or 10-day ethanol exposure and withdrawal.

The next set of experiments aimed to determine whether the cytotoxicity associated with ethanol and pyrithiamine treatment could be prevented by concurrent application of thiamine during the entire pyrithiamine treatment period. Cultures that were treated with 500 μM pyrithiamine and ethanol treatment were co-exposed to thiamine for 10 or 11 days (hydrochloride; Sigma-Aldrich, Co.; 1–100 μM). Thus, cultures were treated with both thiamine and pyrithiamine for 10 or 11 days.

Measurement of cytotoxicity

Cytotoxicity (propidium iodide staining of damaged, dying, or dead neurons and glia) was detected by fluorescent microscopy at 36DIV. The use of propidium iodide as a marker of cytotoxicity significantly correlated with other reliable measures of cell death (for review, see Zimmer et al., 2000) and likely reflected staining of necrotic or end-stage apoptotic cells (Wolbers et al., 2004). Uptake of propidium iodide was visualized with SPOT Advanced version 4.0.2 software for Windows (W. Nuhsbaum Inc., McHenry, IL, USA) using a 2.5× objective on a Leica *DM*IRB microscope (W. Nuhsbaum Inc.) fitted for fluorescence detection (mercury-arc lamp) and connected to a personal computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.). Propidium iodide has a peak excitation wavelength of 536 nm and was excited using a band-pass filter that excited the wavelengths between 515 and 560 nm. The emission of propidium iodide in the visual range is 620 nm.

Intensity of propidium iodide, in arbitrary optical units, was determined by circling the entire cerebellar slice culture using Image J version 1.29x (National Institutes of Health, Bethesda, MD, USA). A background measurement was taken for each culture from the visual field surrounding the slice culture. This procedure allowed for an account of potential daily variation in camera performance. Each experiment was replicated two to four times, which yielded '*n* ' values of 24–48/treatment group. Data from individual experiments were converted to percent of control values using the following formula: $(S-B)/C$, where S was the intensity of fluorescence for a given slice; B was background intensity for that slice; and C was the mean fluorescence in controls. Data from each replicate were then combined for an overall statistical analysis. Control cultures demonstrated an average propidium iodide fluorescence, in arbitrary optical units across all experiments, of 13.455±0.575 (mean±S.E.M.).

Statistical analysis

Data were analyzed using one-way analyses of variance to compare propidium iodide fluorescence in different treatment groups. When appropriate, post hoc analyses were conducted using Fisher's LSD test with a level of significance set at $P<0.05$.

Table 1. Propidium iodide fluorescence in cerebellar cultures withdrawn from 10-day 100 mM ethanol exposure, in cultures treated with ethanol for 11 days, and in cultures exposed to ethanol for 1-day starting at 35DIV

Treatment	Propidium iodide fluorescence
Control cultures	0 (7.74±0.43)
Ethanol withdrawal	4.63±3.25
Continuous ethanol	1.44±5.82
Acute ethanol	5.13±2.33

In all three treatments, no significant effects on propidium iodide uptake were observed when compared to levels demonstrated in control cultures. Data expressed as percentage above untreated controls (mean±S.E.M.). Data in parentheses represent raw fluorescence data expressed in arbitrary units.

RESULTS

Ethanol treatment

One-day of withdrawal from 10-day 100 mM ethanol exposure did not significantly increase the fluorescence of propidium iodide [$F(1,92)=1.341$, $P=0.25$]. The extent of propidium iodide fluorescence in ethanol withdrawn cultures was approximately 5% above untreated control values. Propidium iodide fluorescence in cultures exposed to ethanol for 11-days did not significantly differ from values measured in control cultures [$F(1,46)=1.282$, $P=0.26$]. In addition, one-day ethanol treatment (i.e. acute exposure) starting at 35DIV in previously untreated cultures did not significantly increase propidium iodide fluorescence [$F(1,46)=3.207$, $P=0.08$]. These data are presented in Table 1.

11-Day pyrithiamine exposure and ethanol withdrawal

The next set of experiments examined the potential cytotoxic effects of thiamine deficiency induced by 11-day pyrithiamine (10–500 μM) exposure and ethanol withdrawal. Analyses indicated that 11-day treatment with 100 and 500 μM pyrithiamine significantly increased propidium iodide fluorescence ~21% above levels observed in control tissue [$F(3,66)=5.728$, $P=0.002$; post hoc $P<0.05$]. An ~8% increase above control levels was observed in cultures treated with 10 μM pyrithiamine for 11 days, but this did not reach statistical significance [post hoc $P=0.24$]. Cultures that were withdrawn from 10-day 100 mM ethanol exposure were also treated with pyrithiamine (10–500 μM) for 11 days. When co-applied to cultures during ethanol treatment and withdrawal, pyrithiamine treatment markedly increased propidium iodide fluorescence above control levels [$F(7,112)=3.949$, $P<0.001$; post hoc $P<0.05$]. Post hoc analyses indicated that propidium iodide fluorescence in ethanol withdrawn cultures treated with 100 μM pyrithiamine was significantly elevated ~38% above levels observed in controls. In addition, propidium iodide fluores-

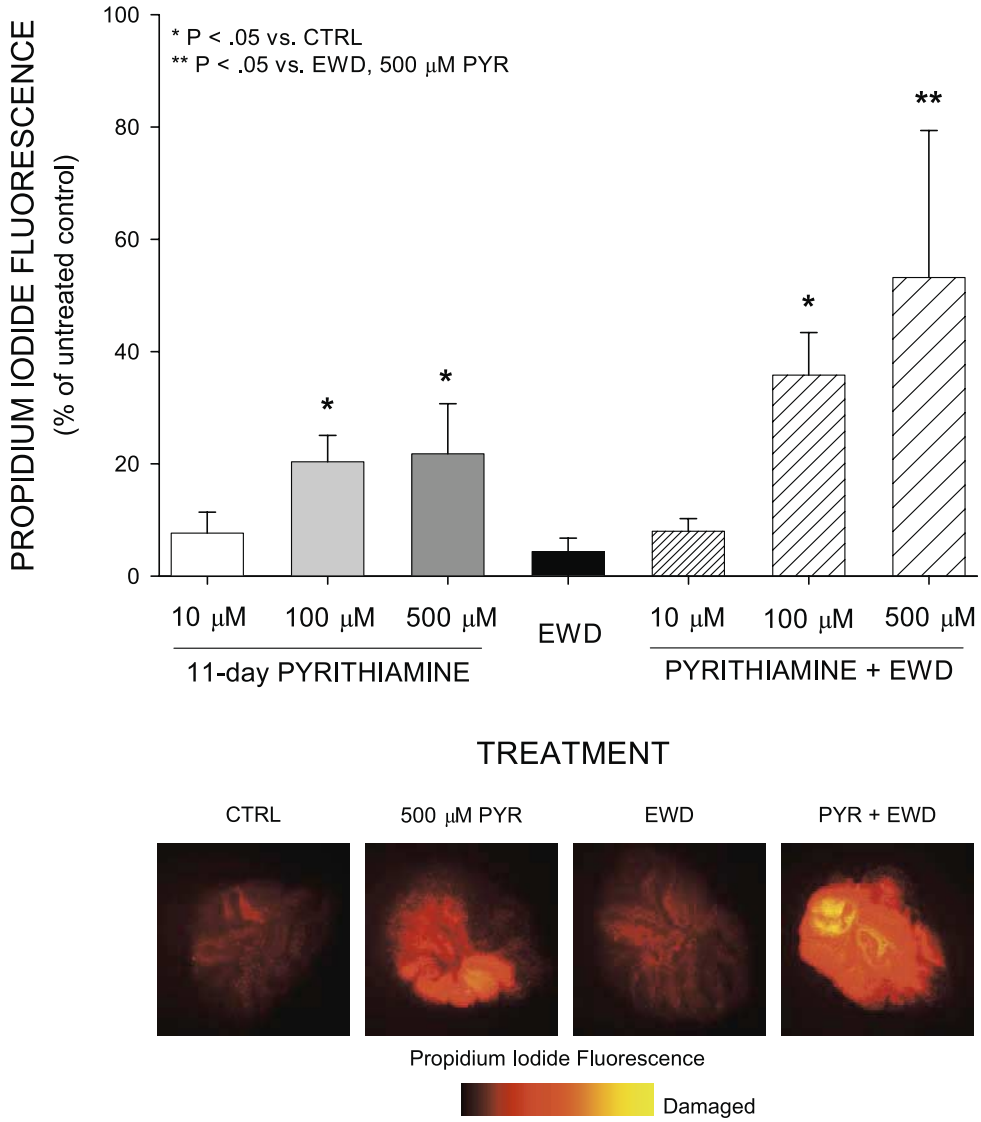

Fig. 1. Effects of 11-day pyrithiamine exposure, ethanol withdrawal, and their combination on propidium iodide fluorescence. Exposure of cultures to pyrithiamine (100–500 μM) resulted in significant uptake of propidium iodide, whereas damage in ethanol withdrawn cultures did not differ from control cultures. When co-applied to cultures during ethanol treatment and withdrawal, 500 μM pyrithiamine exposure markedly increased propidium iodide fluorescence above levels observed in cultures treated with 500 μM pyrithiamine. Data expressed as percentage above untreated control (mean±S.E.M.). Representative images of propidium iodide fluorescence in these cultures are presented at bottom.

cence in ethanol withdrawn cultures treated with 500 μM pyrithiamine were markedly elevated when compared with levels observed in ethanol withdrawn cultures and those treated with 500 μM pyrithiamine alone. These data and representative cerebellar images are presented in Fig. 1.

These experiments examined the potential cytoprotective effects of thiamine treatment against insult induced by 500 μM pyrithiamine and 100 mM ethanol co-exposure. Cultures that were co-exposed to 500 μM pyrithiamine for 11 days and withdrawn from 10-day ethanol were also co-exposed to thiamine (1–100 μM) for 11-days. Analysis revealed that thiamine co-exposure concentration-dependently prevented the elevated propidium iodide fluorescence observed in ethanol withdrawn cultures treated with pyrithiamine [$F(8,99)=3.482$, $P=0.001$; post hoc $P<0.05$]. Post-hoc analyses demonstrated that 10 and 100 μM thiamine significantly reduced propidium iodide fluorescence, with the highest concentration completely preventing the cytotoxicity. Data and representative images of these cultures are presented in Fig. 2.

10-Day pyrithiamine exposure and ethanol withdrawal

Additional cultures were treated with 500 μM pyrithiamine for 10 days with propidium iodide fluorescence measurement at 36DIV. Analyses demonstrated a significant increase (~22%) in these cultures when compared with levels observed in controls [$F(1,42)=6.968$, $P=0.01$; post hoc $P=0.01$]. Pyrithiamine (500 μM) was also co-exposed

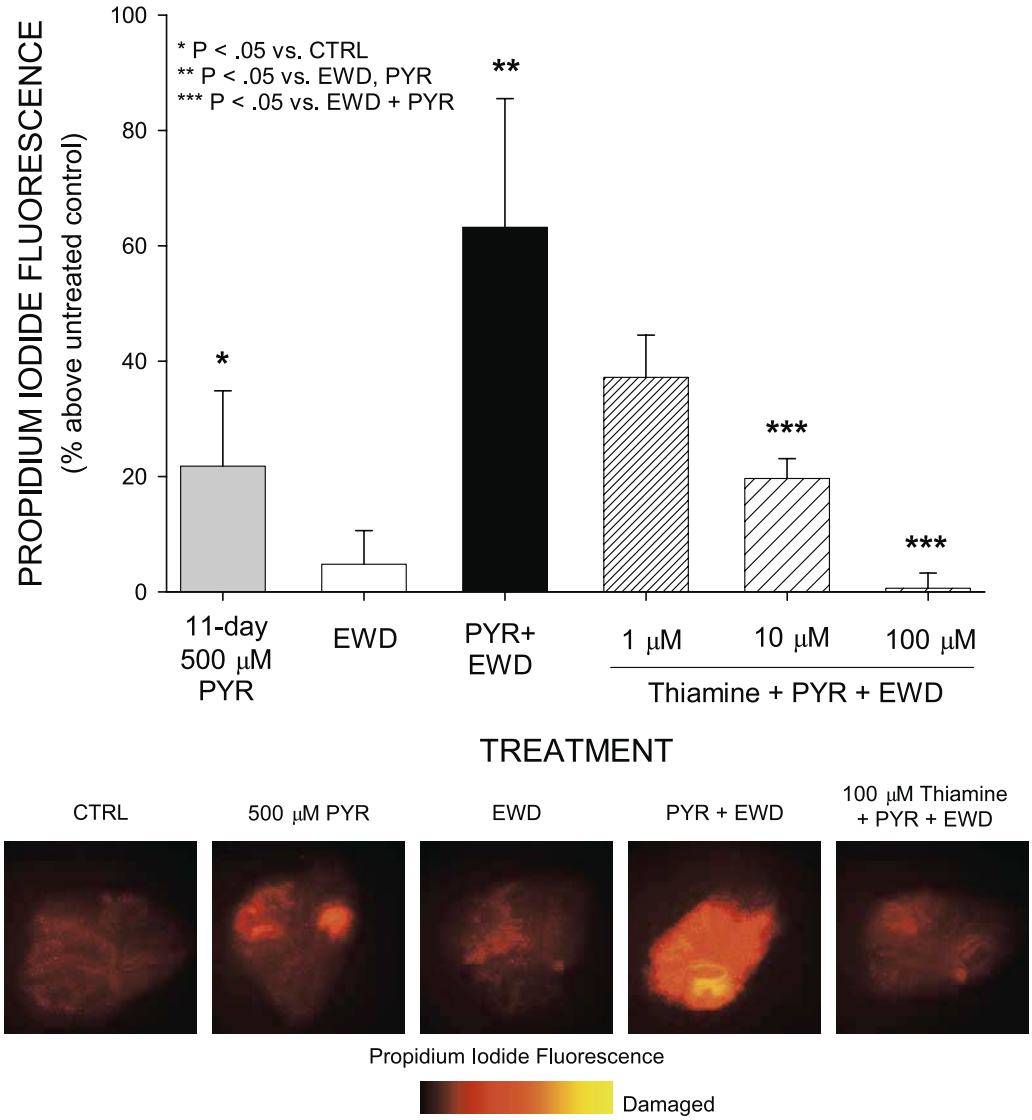

Fig. 2. Effects of 11-day thiamine and 500 μM pyrithiamine co-exposure in cultures withdrawn from ethanol. Thiamine (10–100 μM) co-application with pyrithiamine significantly prevented the significant uptake of propidium iodide observed in cultures withdrawn from ethanol. Data expressed as percentage above untreated control (mean±S.E.M.). Representative images of propidium iodide fluorescence in these cultures are presented at bottom.

to cultures for 10 days with ethanol and withdrawn in the presence of normal culture medium containing propidium iodide. This exposure period resulted in a significant increase in propidium iodide fluorescence above levels observed in cultures withdrawn from ethanol or 10-day 500 μM pyrithiamine exposure alone [$F(7,80)=10.708$, $P<0.001$; post hoc $P<0.05$]. Whereas withdrawal from 10-day ethanol and pyrithiamine treatment increased propidium iodide uptake by ~5% and ~22%, respectively, their co-exposure increased cytotoxicity by ~90% above levels observed in control cultures (Fig. 3).

Subsequently, cultures were co-exposed to thiamine (1–100 μM), pyrithiamine, and ethanol for 10 days and were withdrawn in the presence of normal culture medium containing propidium iodide. Analysis demonstrated that thiamine co-exposure concentration-dependently prevented the cytotoxicity observed in ethanol withdrawn cultures exposed to 10-day pyrithiamine [$F(8,91)=10.254$, $P=0.001$; post hoc $P<0.05$]. Post-hoc analyses revealed that 10 and 100 μM thiamine significantly attenuated the cytotoxicity associated with ethanol and pyrithiamine co-exposure. These data and representative images are presented in Fig. 3.

11-Day pyrithiamine and ethanol exposure

Cultures were then co-exposed to pyrithiamine (10–500 μM) and ethanol for 11 days (Fig. 4). When co-applied to cultures during ethanol treatment, pyrithiamine markedly increased propidium iodide fluorescence above control levels [$F(8,99)=9.624$, $P<0.001$; post hoc $P<0.05$]. Post hoc analyses indicated that propidium iodide fluorescence

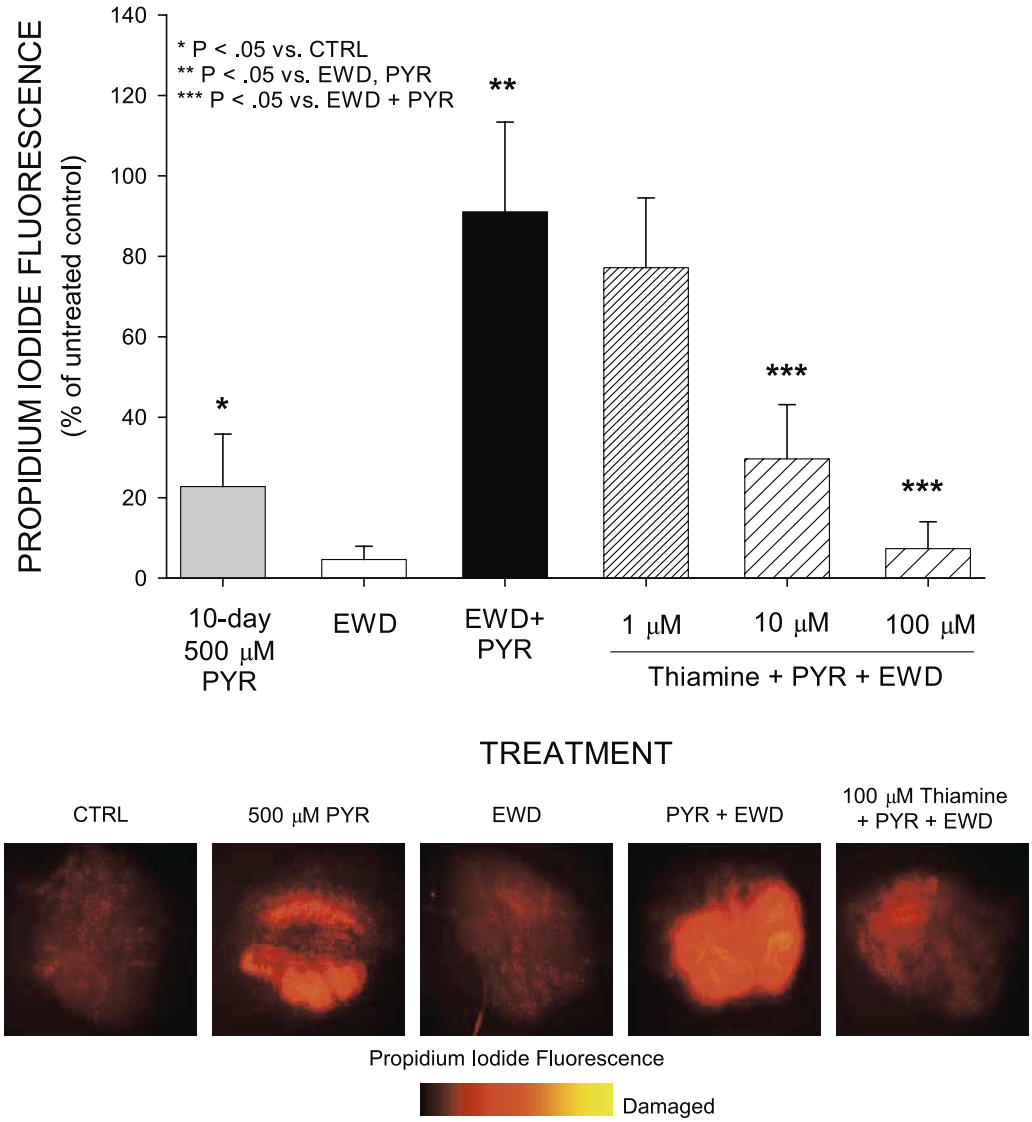

Fig. 3. Propidium iodide fluorescence observed after 10-day thiamine and 500 μM pyrithiamine co-exposure in cultures withdrawn from ethanol. Thiamine (10–100 μM) co-application significantly prevented the significant uptake of propidium iodide observed in cultures treated with pyrithiamine and ethanol for 10 days. Data expressed as percentage above untreated control (mean±S.E.M.). Representative images of propidium iodide fluorescence in these cultures are presented at bottom.

in ethanol-treated cultures co-exposed to 500 μM pyrithiamine was significantly elevated above levels observed in ethanol-treated cultures and in cultures treated with 500 μM pyrithiamine. Additional analyses compared the extent of propidium iodide fluorescence in cultures co-exposed to 11-day pyrithiamine and ethanol with levels observed in cultures treated with 11-day pyrithiamine and withdrawn from 10-day ethanol exposure. Propidium iodide fluorescence between these two groups did not significantly differ [$F(1,68)=1.425$, $P=0.24$].

Finally, cultures were co-exposed to thiamine (1–100 μM), pyrithiamine, and ethanol for 11 days. Analysis indicated that 11-day thiamine co-exposure concentration-dependently prevented the cytotoxicity observed in ethanol- and pyrithiamine-treated cultures [$F(8,99)=9.624$, $P=0.001$; post hoc $P<0.05$]. Post hoc analyses revealed that both 10 and 100 μM thiamine co-exposure significantly prevented ethanol- and pyrithiamine-induced cytotoxicity. These data and representative images of cultures exposed to thiamine, pyrithiamine, and ethanol for 11-days are presented in Fig. 4.

DISCUSSION

Malnutrition in ethanol-dependent individuals has been suggested to be a likely cause of cerebellar damage (Charness, 1993; Diamond and Messing, 1994; Fadda and Rossetti, 1998; Harper and Kril, 1990). Recent studies demonstrated Purkinje cell degeneration and atrophy of the superior vermis in alcoholics (Andersen, 2004; Karhunen et al., 1994; Shear et al., 1996; Sullivan et al., 2000; Torvik and Torp, 1986). The incidence of cerebellar damage is

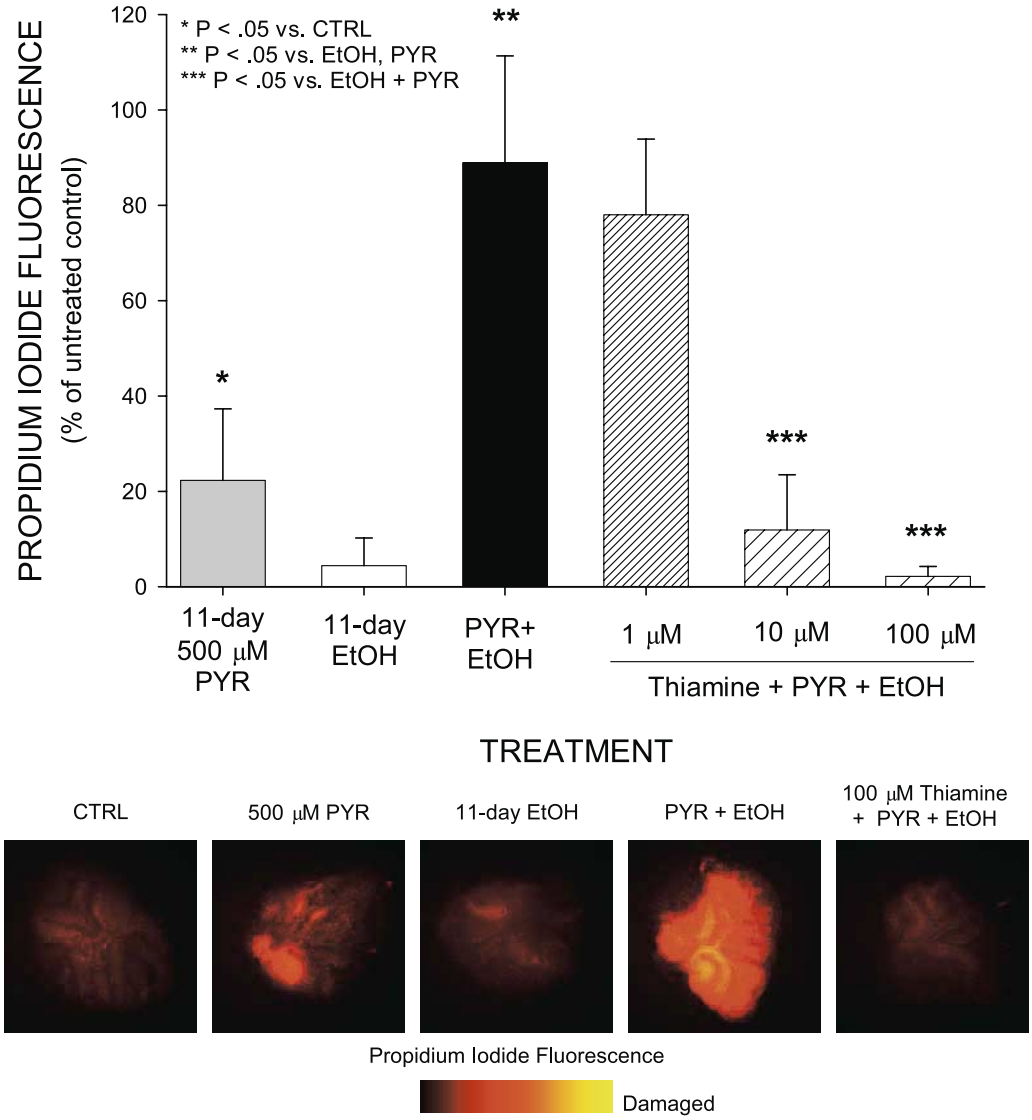

Fig. 4. Uptake of propidium iodide demonstrated after 11-day thiamine, 500 μM pyrithiamine, and continuous ethanol co-exposure. Thiamine (10–100 μM) co-application in cultures treated with pyrithiamine and continuous ethanol significantly prevented the observed cytotoxicity. Data expressed as percentage above untreated control (mean±S.E.M.). Representative images of propidium iodide fluorescence in these cultures are presented at bottom.

more prominent in patients suffering from Wernicke's encephalopathy and thiamine deficiency (Baker et al., 1999; Nicolas et al., 2000; Phillips et al., 1987). However, 45% of well-nourished alcoholics demonstrated cerebellar shrinkage (Nicolas et al., 2000), although past episodes of malnutrition may account for the damage. It remains unclear if thiamine deficiency is a contributing factor underlying chronic ethanol-associated cerebellar damage. Findings from this *in vitro* study suggest that cerebellar damage is related to a pathological interaction between thiamine deficiency and ethanol exposure. The combined exposure of pyrithiamine and chronic ethanol resulted in prominent cerebellar damage compared with each treatment by itself.

In this report, neither continuous 100 mM ethanol exposure nor withdrawal from 10-day ethanol exposure resulted in significant cerebellar uptake of propidium iodide, in contrast to previous findings in hippocampal tissue (Harris et al., 2003; Mulholland et al., 2003; Prendergast et al., 2004). Although the present studies did not assess ethanol concentration in culture medium, these studies employed methods identical to those reported in Prendergast et al. (2004), which included studies measuring ethanol concentration in medium. This study reported that, using a calculated starting concentration of 100 mM ethanol, actual medium concentrations of ethanol were approximately 91 mM on day 1 of treatment and 42 mM on day 5 of treatment, at which time ethanol content was replenished. It is highly unlikely, then, that the lack of cerebellar toxicity observed in the present studies during ethanol treatment or withdrawal can be attributed to treatment with low concentrations of ethanol.

A significant elevation in propidium iodide fluorescence was observed in cultures treated with the thiamine-depleting agent pyrithiamine. Perhaps most important, a marked increase in propidium iodide fluorescence was observed in cultures co-exposed to pyrithiamine and continuous ethanol or those withdrawn from 10-day ethanol. The extent of propidium iodide fluorescence in cultures co-exposed to 11-day pyrithiamine and continuous ethanol was similar to levels observed in cultures treated with 11-day pyrithiamine and withdrawn from 10-day ethanol exposure. It seems as though the ethanol withdrawal phenomenon failed to contribute to the damage associated with ethanol and pyrithiamine co-exposure. In addition, co-exposure of cerebellar cultures to thiamine prevented the cytotoxicity associated with pyrithiamine and ethanol treatment. Interestingly, cerebellar dysfunction may be preventable provided that thiamine administration reversed behavioral impairments, even in actively drinking individuals (Victor et al., 1989), as well as metabolic disturbances of thiamine depletion (Lee et al., 1995). In ethanol-dependent individuals without signs of Wernicke's encephalopathy, thiamine administration dose-dependently increased performance on a cognitive task sensitive to impairments associated with Wernicke's encephalopathy (Ambrose et al., 2001).

Thiamine depletion in alcoholics may be related to a number of factors. First, alcoholics consume less dietary thiamine (Woodhill and Nobile, 1972) and Mg^{2+} (Flink, 1986; Morgan, 1982), which is involved in cellular thiamine utilization. Second, ethanol exposure impaired the absorption of thiamine from the gastrointestinal tract (Gastaldi et al., 1989; Hoyumpa, 1980). Third, both acute and chronic ethanol exposure decreased activity of cellular enzymes responsible for thiamine utilization and increased activity of phosphatases involved in the breakdown of the active form of thiamine (Laforenza et al., 1990; Lavoie and Butterworth, 1995; Poupon et al., 1990; Tallaksen et al., 1992). Finally, there is evidence for a genetic vulnerability to thiamine deficiency (Blass and Gibson, 1977; Martin et al., 1993; Mukherjee et al., 1987). Thus, these means to reduce cellular thiamine may cause neuronal damage through impaired energy metabolism (Pannunzio et al., 2000) or oxidative stress (Todd and Butterworth, 1999). A role for oxidative stress has also been implicated in ethanol-associated neurodegeneration (Fadda and Rossetti, 1998). In addition, damage associated with thiamine deficiency, as well as ethanol withdrawal, may involve increasing glutamatergic processes and over-activity of *N*-methyl-D-aspartate type-glutamate (NMDA) receptors (Langlais, 1995; Prendergast et al., 2004; Whittington et al., 1995).

This report failed to demonstrated significant cerebellar cytotoxicity, as measured by propidium iodide fluorescence, in cultures treated with 100 mM ethanol for 11-days or in cultures withdrawn from 10-day exposure. In animal models, ethanol consumption of 5 weeks or longer followed by a prolonged withdrawal period reduced the number of Purkinje neurons (Jung et al., 2002, 2003; Phillips and Cragg, 1984; Wenisch et al., 1997). In one case, damage was observed in Purkinje neurons following a 4-month withdrawal, but not after a 4-month consumption period (Phillips and Cragg, 1984). Another study demonstrated losses of Purkinje and granule neurons after a 4-month ethanol diet and after a 4-month recovery from this diet (Phillips, 1990). In contrast, a 3-week exposure to ethanol vapor failed to reduce the number of Purkinje neurons (Phillips and Cragg, 1982). Moreover, long-term ethanol consumption by young and aged adult F344 and Wistar-Kyoto rats failed to reduce the number of cerebellar granule neurons or volume of the granule cell layer (Pentney et al., 2002). The intensity of propidium iodide fluorescence in this report was assessed using one measurement for the whole slice across all lobes and zones that accounted for gross damage in the deep cerebellar nuclei (i.e. dentate, interposed, and fastigial nuclei), Purkinje, molecular and granule cell layers, glial cells, etc. Further work in these cultures should examine the specific effects of thiamine deficiency and ethanol exposure on degeneration of Purkinje and granule neurons, deep cerebellar nuclei, and regional differences of cerebellar lobes and functional zones (i.e. superior vermis). In addition, research should address these contradictory findings on the sensitivity of Purkinje and granule cells to ethanol-induced damage.

Some studies have failed to demonstrate hippocampal damage following thiamine deficiency (Langlais, 1992; Langlais et al., 1992), although this is not consistent (Irle and Markowitsch, 1983). These data led to the hypothesis that hippocampal damage in alcoholics is thiamine deficiency-independent (Fadda and Rossetti, 1998), whereas cerebellar damage is related to thiamine deficiency (Charness, 1993; Diamond and Messing, 1994; Fadda and Rossetti, 1998; Harper and Kril, 1990). Data from this laboratory demonstrated a sensitivity to ethanol withdrawal-induced damage in organotypic hippocampal cultures (Harris et al., 2003; Mulholland et al., 2003; Prendergast et al., 2004), but, as presented in this report, ethanol withdrawal failed to significantly increase propidium iodide uptake in organotypic cultures from cerebellum. Significant cerebellar damage was only observed when cultures were chronically co-exposed to pyrithiamine and ethanol. In their review, Fadda and Rossetti (1998) suggest that the sensitivity to ethanol-induced damage in different brain regions may be related to a disparity in subunit composition of glutamate receptors or in the glutamatergic input to that region. Indeed, NMDA receptor binding determined by quantitative autoradiography in human postmortem brain revealed much higher hippocampal binding of glutamate, CGP-39653, and MK-801 when compared with cerebellar vermal binding (Freund and Anderson, 1999). It is possible that this regional difference with regard to ethanol withdrawal toxicity is related to the relatively low density of the ethanol-sensitive NR2B subunit of the NMDA receptor in the cerebellum as compared with high density in the hippocampus (Monyer et al., 1992, 1994). Additionally, hippocampal cultures contain intrinsic glutamatergic projections (i.e. mossy fibers, Shaffer collaterals) that contribute to excitotoxicity (Mulholland and Prendergast, 2003; Prendergast et al., 2004), whereas organotypic culturing techniques removed afferent glutamatergic projections (i.e.

mossy and climbing fibers; Ito, 1984) to the cerebellum. However, intrinsic glutamate-enriched parallel fibers, the axons of cerebellar granule cells, do synapse on Purkinje cells (Somogyi et al., 1986; Ottersen, 1987). Following a 3- or 4-day ethanol exposure, both hippocampal slice cultures and cerebellar granule cells demonstrated an increased response to glutamatergic insult (Hoffman, 1995; Iorio et al., 1993; Mayer et al., 2002). Thus, further *in vivo* and *in vitro* work should examine the combined effect of thiamine deficiency and ethanol exposure to properly compare the mechanisms of ethanol-associated damage between the cerebellum and hippocampus.

The high affinity thiamine transporter in neurons has a K_d of ~40 nM for thiamine and ~60 nM for pyrithiamine (Bettendorff and Wins, 1994). It should be noted that the thiamine concentration in the culture medium utilized in this model was ~2 μM. Because this organotypic model allows for a slow diffusion of drug from medium through the biopore membrane into tissue, it was necessary to use concentrations of compounds (e.g. 500 μM pyrithiamine and 100 μM thiamine) above the K_d for the high affinity transporter and above concentrations used in models employing dissociated cells. To compare, cell death was observed in dissociated cerebellar granule cells after a 7-day exposure to 50 μM pyrithiamine (Pannunzio et al., 2000). Although the concentrations employed in this study are well above the K_d for the high affinity transporter, it remains that thiamine, at concentrations (i.e. ≥10 μM) lower than pyrithiamine, effectively reduced the elevation of propidium iodide uptake observed in cultures treated with 500 μM pyrithiamine and ethanol.

After a 10-week thiamine-deficient diet or ethanol vapor exposure, no significant cytotoxicity was observed in mouse cerebellum; however, when given a combined thiamine-deficient diet with ethanol, axon terminal degeneration was present in deep cerebellar nuclei (Phillips, 1987). Additionally, a synergic effect on reference memory was demonstrated in rats exposed chronically to thiamine deficiency and ethanol treatment (Ciccia and Langlais, 2000). Taken in conjunction with findings from the above *in vivo* studies, results from this report suggest an interaction between ethanol exposure and thiamine deficiency in ethanol-associated cerebellar damage. In conclusion, these data imply that cerebellar dysfunction in ethanol-dependent individuals may be related to thiamine deficiency. In accordance with studies that observed a beneficial effect of thiamine administration on cerebellar function (Ambrose et al., 2001; Lee et al., 1995; Victor et al., 1989), this study demonstrated that thiamine administration prevented the cytotoxic effects of thiamine deficiency and ethanol co-exposure.

Acknowledgments—The authors acknowledge the generous support of NIAAA (AA014771-P.J.M.; AA13561-M.A.P.). The authors also thank Eva Kaplan, Robert Holley, Allison Hensley, and Alexandra Kowalski for their exceptional technical assistance in completing these studies.

REFERENCES

Adeyinka AO, Akinyinka OO, Falade AG (1996) Computerized tomography measures of brain slice area and ventricular sizes in protein energy malnutrition: a preliminary study. West Afr J Med 15:232–236.

Adinoff B, Iranmanesh A, Veldhuis J, Fisher J (1998) Disturbances of stress response: the role of the HPA axis during alcohol withdrawal and abstinence. Alcohol Health Res World 22:67–72.

Ambrose ML, Bowden SC, Whelan G (2001) Thiamin treatment and working memory function of alcohol-dependent people: preliminary findings. Alcohol Clin Exp Res 25:112–116.

Andersen BB (2004) Reduction of Purkinje cell volume in cerebellum of alcoholics. Brain Res 8:10–18.

Baker KG, Harding AJ, Halliday GM, Kril JJ, Harper CG (1999) Neuronal loss in functional zones of the cerebellum of chronic alcoholics with and without Wernicke's encephalopathy. Neuroscience 91:429–438.

Bettendorff L, Wins P (1994) Mechanism of thiamine transport in neuroblastoma cells. Inhibition of a high affinity carrier by sodium channel activators and dependence of thiamine uptake on membrane potential and intracellular ATP. J Biol Chem 269:4379–4385.

Blank NK, Vick NA, Schulman S (1975) Wernicke's encephalopathy: an experimental study in the rhesus monkey. Acta Neuropathol 31:137–150.

Blass JP, Gibson GE (1977) Abnormality of a thiamine-requiring enzyme in patients with Wernicke-Korsakoff syndrome. N Engl J Med 297:1367–1370.

Butterworth RF, Heroux M (1989) Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. J Neurochem 52:1079–1084.

Casirola D, Ferrari G, Gastaldi G, Patrini C, Rindi G (1988) Transport of thiamine by brush-border membrane vesicles from rat small intestine. J Physiol 398:329–339.

Casirola D, Patrini C, Ferrari G, Rindi G (1990) Thiamine transport by human erythrocytes and ghosts. J Membr Biol 188:11–18.

Charness ME (1993) Brain lesions in alcoholics. Alcohol Clin Exp Res 17:2–11.

Ciccia RM, Langlais PJ (2000) 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 24:622–634.

Collins RC, Kirkpatrick JB, McDougal DB (1970) Some regional pathological and metabolic consequences in mouse brain of pyrithiamine-induced thiamine deficiency. J Neuropathol Exp Neurol 29:57–69.

Diamond I, Messing RO (1994) Neurologic effects of alcoholism. West J Med 161:279–287.

Duka T, Gentry J, Malcolm R, Ripley TL, Borlikova G, Stephens DN, Veatch LM, Becker HC, Crews FT (2004) Consequences of multiple withdrawals from alcohol. Alcohol Clin Exp Res 28:233–246.

Eckardt MJ, Martin PR (1986) Clinical assessment of cognition in alcoholism. Alcohol Clin Exp Res 10:123–127.

Fadda F, Rossetti ZL (1998) Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog Neurobiol 56:385–431.

Flink EB (1986) Magnesium deficiency in alcoholism. Alcohol Clin Exp Res 10:590–594.

Freund G, Anderson KJ (1999) Glutamate receptors in the cingulated cortex, hippocampus, and cerebellar vermis of alcoholics. Alcohol Clin Exp Res 23:1–6.

Gastaldi G, Casirola D, Ferrari G, Rindi G (1989) Effect of chronic ethanol administration on thiamine transport in microvillous vesicles of rat small intestine. Alcohol Alcohol 24:83–89.

Gibson G, Nielsen P, Mykytyn V, Carlson K, Blass J (1989) Regionally selective alterations in enzymatic activities and metabolic fluxes during thiamin deficiency. Neurochem Res 14:17–24.

Grant BF, Dawson DA, Stinson FS, Chou SP, Dufour MC, Pickering RP (2004) The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991–1992 and 2001–2002. Drug Alcohol Depend 74:223–234.

Harper CG, Kril JJ (1990) Neuropathology of alcoholism. Alcohol Alcohol 25:207–216.

Harris BR, Gibson DA, Prendergast MA, Blanchard JA, Holley RC, Hart SR, Scotland RL, Foster TC, Pedigo NW, Littleton JM (2003) The neurotoxicity induced by ethanol withdrawal in mature organotypic hippocampal slices might involve cross-talk between metabotropic glutamate type 5 receptors and N-methyl-D-aspartate receptors. Alcohol Clin Exp Res 27:1724–1735.

Hoffman PL (1995) Glutamate receptors in alcohol withdrawal-induced neurotoxicity. Metab Brain Dis 10:73–79.

Hommer D, Momenan R, Kaiser E, Rawlings R (2001) Evidence for a gender-related effect of alcoholism on brain volumes. Am J Psychiatry 158:198–204.

Hoyumpa AM Jr (1980) Mechanisms of thiamin deficiency in chronic alcoholism. Am J Clin Nutr 33:2750–2761.

Hunt WA (1993) Are binge drinkers more at risk of developing brain damage?Alcohol 10:559–561.

Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996) Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press.

Iorio KR, Tabakoff B, Hoffman PL (1993) Glutamate-induced neurotoxicity is increased in cerebellar granule cells exposed chronically to ethanol. Eur J Pharmacol 248:209–212.

Irle E, Markowitsch HJ (1983) Widespread neuroanatomical damage and learning deficits following chronic alcohol consumption or vitamin-B1 (thiamine) deficiency in rats. Behav Brain Res 9:277–294.

Ito M (1984) The cerebellum and neural control. New York: Raven Press.

Iwashima A, Wakabayashi Y, Nose Y (1975) Thiamine transport mutants of Saccharomyces cerevisiae. Biochim Biophys Acta 413:243–247.

Jacobson RR, Lishman WA (1990) Cortical and diencephalic lesions in Korsakoff's syndrome: a clinical and CT scan study. Psychol Med 20:63–75.

Jung ME, Watson DG, Wen Y, Simpkins JW (2003) Role of protein kinase C in estrogen protection against apoptotic cerebellar cell death in ethanol-withdrawn rats. Alcohol 31:39–48.

Jung ME, Yang SH, Brun-Zinkernagel AM, Simpkins JW (2002) Estradiol protects against cerebellar damage and motor deficit in ethanol-withdrawn rats. Alcohol 26:83–93.

Karhunen PJ, Erkinjuntti T, Laippala P (1994) Moderate alcohol consumption and loss of cerebellar Purkinje cells. BMJ 308:1663–1667.

Kohn MR, Ashtari M, Golden NH, Schebendach J, Patel M, Jacobson MS, Shenker IR (1997) Structural brain changes and malnutrition in anorexia nervosa. Ann N Y Acad Sci 28:398–399.

Laforenza U, Patrini C, Gastaldi G, Rindi G (1990) Effects of acute and chronic ethanol administration on thiamine metabolizing enzymes in some brain areas and in other organs of the rat. Alcohol Alcohol 25:591–603.

Langlais (1992) Role of diencephalic lesions and thiamine deficiency in Korsakoff's amnesia: insights from animal models. In: Neuropsychology of memory (Squire LR, Butters N, eds), pp 440–450. New York: Guilford Press.

Langlais PJ (1995) Pathogenesis of diencephalic lesions in an experimental model of Wernicke's encephalopathy. Metab Brain Dis 10:31–44.

Langlais PJ, Mandel RJ, Mair RG (1992) Diencephalic lesions, learning impairments, and intact retrograde memory following acute thiamine deficiency in the rat. Behav Brain Res 48:1777–1785.

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

Lavoie J, Butterworth RF (1995) Reduced activities of thiamine-dependent enzymes in brains of alcoholics in the absence of Wernicke's encephalopathy. Alcohol Clin Exp Res 19:1073–1077.

Lee H, Tarter J, Holburn GE, Price RR, Weinstein DD, Martin PR (1995) In vivo localized proton NMR spectroscopy of thiamine-deficient rat brain. Magn Reson Med 34:313–318.

Lieber CS (2003) Relationships between nutrition, alcohol use, and liver disease. Alcohol Res Health 27:220–231.

Mann K, Agartz I, Harper C, Shoaf S, Rawlings RR, Momenan R, Hommer DW, Pfefferbaum A, Sullivan EV, Anton RF, Drobes DJ, George MS, Bares R, Machulla HJ, Mundle G, Reimold M, Heinz A (2001) Neuroimaging in alcoholism: ethanol and brain damage. Alcohol Clin Exp Res 25:104S–109S.

Martin PR, McCool BA, Singleton CK (1993) Genetic sensitivity to thiamine deficiency and development of alcoholic organic brain disease. Alcohol Clin Exp Res 17:31–37.

Martin PR, Singleton CK, Hiller-Sturmhofel S (2003) The role of thiamine deficiency in alcoholic brain disease. Alcohol Res Health 27:134–142.

Mayer S, Harris BR, Gibson DA, Blanchard JA, Prendergast MA, Holley RC, Littleton J (2002) Acamprosate, MK-801, and ifenprodil inhibit neurotoxicity and calcium entry induced by ethanol withdrawal in organotypic slice cultures from neonatal rat hippocampus. Alcohol Clin Exp Res 26:1468–1478.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529–540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217–1221.

Morgan MY (1982) Alcohol and nutrition. Br Med Bull 38:21–29.

Mukherjee AB, Svoronos S, Ghazanfari A, Martin PR, Fisher A, Roecklein B, Rodbard D, Staton R, Behar D, Berg CJ, Manjunath R (1987) Transketolase abnormality in cultured fibroblasts from familial chronic alcoholic men and their male offspring. J Clin Invest 79:1039–1043.

Mulholland PJ, Harris BR, Wilkins LH, Self RL, Blanchard JA, Holley RC, Littleton JM, Prendergast MA (2003) Opposing effects of ethanol and nicotine on hippocampal calbindin-D28k expression. Alcohol 31:1–10.

Mulholland PJ, Prendergast MA (2003) Transection of intrinsic polysynaptic pathways reduces N-methyl-D-aspartate neurotoxicity in hippocampal slice cultures. Neurosci Res 46:369–376.

Mulholland PJ, Self RL, Harris BR, Little HJ, Littleton JM, Prendergast MA (2005) Corticosterone exposure increases cytosolic calcium accumulation and neurotoxicity associated with ethanol withdrawal in rat hippocampal slice cultures. Alcohol Clin Exp Res 27:871–881.

Nicolas JM, Fernandez-Sola J, Robert J, Antunez E, Cofan M, Cardenal C, Sacanella E, Estruch R, Urbano-Marquez A (2000) High ethanol intake and malnutrition in alcoholic cerebellar shrinkage. QJM 93:449–456.

Ottersen OP (1987) Postembedding light- and electron microscopic immunocytochemistry of amino acids: description of a new model system allowing identical conditions for specificity testing and tissue processing. Exp Brain Res 69:167–174.

Pannunzio P, Hazell AS, Pannunzio M, Rao KV, Butterworth RF (2000) Thiamine deficiency results in metabolic acidosis and energy failure in cerebellar granule cells: an in vitro model for the study of cell death mechanisms in Wernicke's encephalopathy. J Neurosci Res 62:286–292.

Pentney RJ, Mullan BA, Felong AM, Dlugos CA (2002) The total numbers of cerebellar granule neurons in young and aged Fischer 344 and Wistar-Kyoto rats do not change as a result of lengthy ethanol treatment. Cerebellum 1:79–89.

Phillips SC (1987) Neuro-toxic interaction in alcohol-treated, thiamine-deficient mice. Acta Neuropathol 73:171–176.

Phillips SC (1990) Cerebellar white matter after long-term ethanol consumption in mice. J Stud Alcohol 51:14–18.

Phillips SC, Cragg BG (1982) A change in susceptibility of rat cerebellar Purkinje cells to damage by alcohol during fetal, neonatal and adult life. Neuropathol Appl Neurobiol 8:441–454.

Phillips SC, Cragg BG (1984) Alcohol withdrawal causes a loss of cerebellar Purkinje cells in mice. J Stud Alcohol 45:475–480.

Phillips SC, Harper C, Kril J (1987) A quantitative histological study of the cerebellar vermis in alcoholic patients. Brain 110:301–314.

Poupon RE, Gervaise G, Riant P, Houin G, Tillement JP (1990) Blood thiamine and thiamine phosphate concentrations in excessive drinkers with or without peripheral neuropathy. Alcohol Alcohol 25:605–611.

Prendergast MA, Harris BR, Mulholland PJ, Blanchard JA 2nd, Gibson DA, Holley RC, Littleton JM (2004) Hippocampal CA1 region neurodegeneration produced by ethanol withdrawal requires activation of intrinsic polysynaptic hippocampal pathways and function of N-methyl-D-aspartate receptors. Neuroscience 124:869–877.

Reuler JB, Girard DE, Cooney TG (1985) Current concepts. Wernicke's encephalopathy. N Engl J Med 312:1035–1039.

Schoffeniels E (1990) Molecular aspects of bioelectrogenesis. Arch Int Physiol Biochem 97:647–662.

Shear PK, Sullivan EV, Lane B, Pfefferbaum A (1996) Mammillary body and cerebellar shrinkage in chronic alcoholics with and without amnesia. Alcohol Clin Exp Res 20:1489–1495.

Somogyi P, Halasy K, Somogyi J, Storm-Mathisen J, Ottersen OP (1986) Quantification of immunogold labelling reveals enrichment of glutamate in mossy and parallel fibre terminals in cat cerebellum. Neuroscience 19:1045–1050.

Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173–182.

Sullivan EV, Deshmukh A, Desmond JE, Lim KO, Pfefferbaum A (2000) Cerebellar volume decline in normal aging, alcoholism, and Korsakoff's syndrome: relation to ataxia. Neuropsychology 14:341–352.

Tallaksen CM, Bohmer T, Bell H (1992) Blood and serum thiamin and thiamin phosphate esters concentrations in patients with alcohol dependence syndrome before and after thiamin treatment. Alcohol Clin Exp Res 16:320–325.

Tarter RE, Hegedus AM, van Thiel DH, Gavaler JS, Schade RR (1986) Hepatic dysfunction and neuropsychological test performance in alcoholics with cirrhosis. J Stud Alcohol 47:74–77.

Todd K, Butterworth RF (1999) Mechanisms of selective neuronal cell death due to thiamine deficiency. Ann N Y Acad Sci 893:404–411.

Torvik A, Torp S (1986) The prevalence of alcoholic cerebellar atrophy. A morphometric and histological study of an autopsy material. J Neurol Sci 75:43–51.

van Thiel DH, Lester R (1978) Further evidence for hypothalamic-pituitary dysfunction in alcoholic men. Alcohol Clin Exp Res 2:265–270.

Victor M, Davis RD, Collins GH (1989) The Wernicke-Korsakoff syndrome and related neurologic disorders due to alcoholism and malnutrition. Philadelphia, PA: FA Davis Co.

Wenisch S, Hild A, Bitsch I, Leiser R, Hummel G (1997) Ultrastructure of rat Purkinje neurons after chronic ethanol consumption and prolonged abstinence. Anat Histol Embryol 26:93–98.

Whittington MA, Lambert JDC, Little HJ (1995) Increased NMDA-receptor and calcium channel activity during ethanol withdrawal hyperexcitability. Alcohol Alcohol 30:105–114.

Wolbers F, Buijtenhuijs P, Haanen C, Vermes I (2004) Apoptotic cell death kinetics in vitro depend on the cell types and inducers used. Apoptosis 9:385–392.

Woodhill JM, Nobile S (1972) Thiamine in the 1970 Australian diet with special reference to cereals and the assessment of thiamine status. Int J Vitam Nutr Res 42:435–443.

World Health Organization (2001) The World Health Report, 2001. Geneva, Switzerland: World Health Organization.

Yoshioka K (1984) Some properties of the thiamine uptake system in rat hepatocytes. Biochim Biophys Acta 778:201–209.

Zimmer J, Kristensen BW, Jakobsen B, Noraberg J (2000) Excitatory amino acid neurotoxicity and modulation of glutamate receptor expression in organotypic brain slice cultures. Amino Acids 19:7–21.

(Accepted 22 June 2005)
(Available online 13 September 2005)