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Changes in Some Pro- and Antioxidants in Rat Cerebellum After Chronic Alcohol Intake

Hélène Rouach, Pascal Houzé, Monique Gentil, Marie-Thérèse Orfanelli and Roger Nordmann*

DEPARTMENT OF BIOMEDICAL RESEARCH ON ALCOHOLISM, BIOMÉDICALE DES SAINTS-PÈRES, 45 RUE DES SAINTS-PÈRES, 75270 PARIS CEDEX 06, FRANCE

ABSTRACT. Some pro- and antioxidants were measured in the cerebellum from ethanol-fed rats using ethanol administration in drinking water as a model of moderate alcohol intoxication. After 4 weeks of ethanol intake, a 30% increase in the nonheme iron content in the cerebellum occurred in ethanol-fed rats as compared to control animals. The low-molecular-weight chelated iron (LMWC-Fe) content as well as the percentage of total nonheme iron represented by LMWC-Fe were increased in the cerebellar cytosol after chronic ethanol administration. Cerebellar copper and selenium concentrations were lower and zinc concentration higher in ethanol-fed rats than in controls. Ethanol consumption decreased the cerebellar vitamin E level. Glutathione S-transferase [EC 2. 5. 1. 18] activity was higher, whereas glutathione peroxidase [glutathione: H_2O_2 oxidoreductase, EC 1. 11. 1. 9] activity was not altered by ethanol treatment. No significant changes in cerebellar lipid peroxidation, carbonyl protein content, or glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming) EC 6. 3. 1. 2] activity were observed. These results suggest that adaptative increases in some elements of the antioxidant defense may counteract the increase in LMWC-Fe, a pro-oxidant factor, and prevent the occurrence of overt cellular lipid and protein damage. However, after 8 weeks of ethanol intake, the activity of glutamine synthetase, an enzyme specially sensitive to inactivation by oxygen radicals, was decreased, suggesting that this prevention was not totally achieved. BIOCHEM PHARMACOL 53;4:539–545, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ethanol; cerebellum; LMWC-iron; trace elements; antioxidants; oxidative modification

It is well known that long-term alcohol consumption in man can result in cognitive and motor deficits [1]. One of the brain regions that appears highly sensitive to alcohol exposure is the cerebellum. Changes in the firing properties of Purkinje cells, structural alterations, and cell loss have been repeatedly reported in the cerebellum of developing, as well as adult, rodents following chronic alcohol exposure [2–4].

However, the mechanisms linking chronic ethanol exposure to brain damage are not yet clearly understood. Free radical mechanisms, which have been implicated in the pathogenesis of neurodegenerative conditions such as aging [5], Parkinson's and Alzheimer's disease, as well as Down's syndrome [6, 7], could play a prominent role in the disturbances induced in the brain by chronic ethanol administration.

One may recall that Seligman et al. [8] observed that lipid peroxidation was enhanced in spinal cord following minimal physical trauma in ethanol-pretreated cats, whereas the same trauma did not induce any change in lipid peroxidation in control cats not pretreated with ethanol. Our group reported later that acute ethanol, without any associated trauma, can induce an enhanced lipid peroxidation (2 and 4 hr after the i.p. administration of 2.3 g ethanol/kg b.wt.) in rat cerebellum [9, 10]. The cerebellum was selected in these studies because of its well-known sensitivity to ethanol-induced dysfunction. Furthermore, among all the brain regions, the cerebellum shows the lowest concentration of α-tocopherol [11], the most important chain-breaking antioxidant. We later reported [12] that, in addition to resulting in enhanced lipid peroxidation, an acute ethanol load elicited at the cerebellar level an increase in the total nonheme iron level and in the cytosolic low-molecular chelated iron compounds (i.e. in the fraction of iron that is active as catalyst of free radical mechanisms and can also be defined as loosely bound or "free" iron). These disturbances were accompanied by a decrease in cerebellar α-tocopherol [10], zinc, copper, and selenium [13] contents.

The present work was undertaken to establish if moderate chronic ethanol intake also elicits disturbances in some cerebellar pro- and antioxidants and if such disturbances may affect important cellular targets. The conditions of ethanol administration used (10%, v/v, ethanol as sole drinking fluid) have been previously considered as representing "a reasonable model for studies of the effect of

* Corresponding author. Dr. Hélène Rouach, Department of Biomedical Research on Alcoholism, Biomédicale des Saints-Pères, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France. Tel. (33) 1-42-86-22-21; FAX (33) 1-42-86-04-02.

† *Abbreviations:* LMWC-Fe, low-molecular-weight chelated iron; GPx, glutathione peroxidase; GST, glutathione S-transferase; GS, glutamine synthetase.

moderate alcohol consumption on specific biochemical pathways" [14]. As emphasized by Lieber et al. [15], the shortcomings of this model are represented by the impossibility of obtaining sustained appreciable levels of ethanol in the blood. However, this model presents the huge advantage of being closer to the usual human conditions of alcohol intake than the liquid diet or the inhalation techniques of ethanol administration.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were maintained on a standard laboratory diet (Iffa-Credo, U.A.R., Lyon, France) containing 64% of energy as carbohydrate, 11% as lipid, and 25% as protein. The iron, zinc, copper, and selenium content of the diet was 33, 10, 3.3, 0.16 mg/1000 cal., respectively.

Chronic ethanol-treated rats weighing *ca.* 100 g at the start of the experiment were fed the basal diet *ad lib.* and received an aqueous ethanol solution (10%, v/v) as sole drinking fluid. The average ethanol intake was 1.41 ± 0.18, 1.61 ± 0.08, 1.77 ± 0.11, and 1.77 ± 0.15 g/rat/day from the first to the fourth week, respectively ($n = 6$ in each group). The ethanol thus represented approximately 18% of the total energy intake. Control rats were given tap water as drinking fluid.

The growth rate of the ethanol-fed rats (8 g/day) did not differ significantly from that of the control animals. This finding is in accordance with previous reports from other groups [14, 16]. To compensate for the 18% of total energy represented by the alcohol intake, the spontaneous solid food intake was reduced in the ethanol-fed rats compared to the control animals. However, this reduction does not apparently result in nutritional deficiencies, a finding that is not surprising, given the amount of micronutriments in the diet used were far in excess of the levels recommended by the American Institute of Nutrition [17]. The vitamin E content of the diet (57 IU/1000 cal.) was also much larger than the recommended amount.

At the end of the experimental period (4 weeks when not otherwise stated), the rats fasted for 16 hr. However, access to the ethanol drinking fluid was maintained to prevent any possible withdrawal stress. Blood was collected at 10 a.m. for ethanol determination and the animals were killed by decapitation. The cerebellum was rapidly removed, washed, mopped up, and either immediately used for the preparation of subcellular fractions or frozen and kept in liquid nitrogen until used for the analysis of trace elements and vitamin E. As stated in the text, some experiments were undertaken in rats in which the ethanol feeding period was extended to 8 weeks.

Homogenization and Subcellular Fractionation

The cerebellum was homogenized either in 9 vol. ice-cold sucrose (0.33 M, pH 7.4) for the determination of nonheme iron in subcellular fractions or in a solution (pH 7.4) containing sucrose (0.33 M), EDTA (3 mM), Tris (20 mM) for the determination of GPx† and GST activities or in Hepes (10 mM, pH 7.4) containing NaCl (137 mM), KCl (4.6 mM), KH_2PO_4 (1.1 mM), Mg SO_4 (0.6 mM), leupeptin (0.5 μg/mL), pepstatin (0.7 μg/mL), phenyl methyl sulfonyl fluoride (40 μg/mL), aprotinin (0.5 μg/mL), and EDTA (1.1 mM) for determination of protein carbonyl content and GS activity.

The total cerebellar homogenate was submitted to differential centrifugation by a 5-fraction scheme according to Laduron *et al.* [18]. Nuclei, heavy mitochondria, light mitochondria, microsomes, and cytosol prepared by this procedure were used for the determination of the nonheme iron content. Cytosolic fractions were used for the determination of GPx and GST activities.

The supernatant used for the determination of protein carbonyl and GS activity was recovered after centrifugation of cerebellar homogenates at 100,000 g for 5 min at 4°C.

Preparation of Low-molecular-weight Filtrates

Preparation of low-molecular-weight filtrates was achieved by filtration of samples of the cerebellar cytosolic fractions through a YMT ultrafiltration membrane in an Amicon-MPS1 device (cut-off 30,000 daltons) [19].

Assay of Enzymatic Activities

Selenium-dependent and total GPx activities were assayed essentially as described by Paglia and Valentine [20], using H_2O_2 (0.125 mM) and cumene hydroperoxide (CuOOH) (1.5 mM) as substrate, respectively. GST activity was measured using 1-chloro-2-4-dinitrobenzene (1 mM) as described by Habig *et al.* [21]. GS activity was determined essentially as described by Rowe *et al.* [22].

α-Tocopherol

α-Tocopherol was determined by HPLC following Vatassery and Hagen [23].

Trace Elements

Nonheme iron and LMWC-Fe were determined after trichloracetic/hydrochloric acid extraction by inductively coupled plasma atomic emission spectrometry [24]. Copper and zinc were determined by flame atomic absorption spectrometry after acidic extraction, and selenium was measured by furnace graphite atomic spectrometry with Zeemann correction. All solutions were prepared with Chelex 100-treated water.

Protein Carbonyl Content

The protein carbonyl content was determined spectrophotometrically using the 2,4-dinitrophenylhydrazine labeling procedure described in [25].

Rate of Lipid Peroxidation

A sample of cerebellum was homogenized (1:40) in phosphate buffer (20 mM, pH 7.4), centrifuged, and incubated without added substrates for 30 min at 37°C in a shaking water bath. Aliquots of the incubated mixture were taken every 5 min to determine the lipid peroxidation rate. Lipid peroxidation was evaluated from the formation of thiobarbituric acid-reactive substances according to Mihara *et al.* [26].

Protein was measured according to Lowry et al. [27].

Ethanol was measured in blood by gas chromatography according to Cullen et al. [28].

Expression of Results

All results are given as means ± SEM, statistical analysis being performed by the Student's t test.

RESULTS

The blood ethanol level measured before sacrifice in 4-week ethanol-fed rat was 7.8 ± 0.5 mM.

As shown in Fig. 1, the cerebellar nonheme iron content was significantly increased in ethanol-fed rats. This increase was apparent in all the subcellular fractions studied, except the crude nuclear fraction. The magnitude of the increase in heavy mitochondrial, light mitochondrial, microsomal, and cytosolic preparations was 19, 27, 55, and 29%, respectively. Furthermore, a 55% increase in the cerebellar cytosolic LMWC-iron content, as well as a higher percentage of cytosolic nonheme iron represented by LMWC-iron, were found in the cerebellum of ethanol-treated rats (Table 1).

The cerebellar zinc content was increased (+15%) in ethanol-treated rats, whereas the copper and selenium contents were significantly decreased (by 26.7 and 35%, respectively) (Table 2). The cerebellar α-tocopherol content was also decreased (−7%) after ethanol feeding (Table 2).

The cerebellar cytosolic activities of the selenium-dependent GPx (measured with H_2O_2 as substrate) and total GPx (measured with CuOOH) were unaffected by chronic ethanol feeding, whereas the GST activity was increased (+33%) after ethanol feeding (Table 3). The increase in GST activity was still present after 8 weeks of ethanol feeding (134 ± 10 vs. 111 ± 14 nmol/min/mg protein in control rats; $p < 0.02$; $n = 5$ in each group).

The cerebellar lipid peroxidation rate (studied during incubation of tissue homogenates without addition of any inducer) was not significantly different in 4-week ethanol-treated and control animals. The same held true for the protein carbonyl content and GS activity (Table 4). GS activity was, however, decreased in rats fed ethanol for 8 weeks (0.48 ± 0.04 vs. 0.57 ± 0.05 nmol/min/mg protein in control animals; $p < 0.02$; $n = 11$ in each group).

DISCUSSION

The present results show that chronic ethanol administration elicits an increase in the nonheme iron content in the cerebellum. Several mechanisms may be considered as being responsible for iron accumulation in the cerebellum of

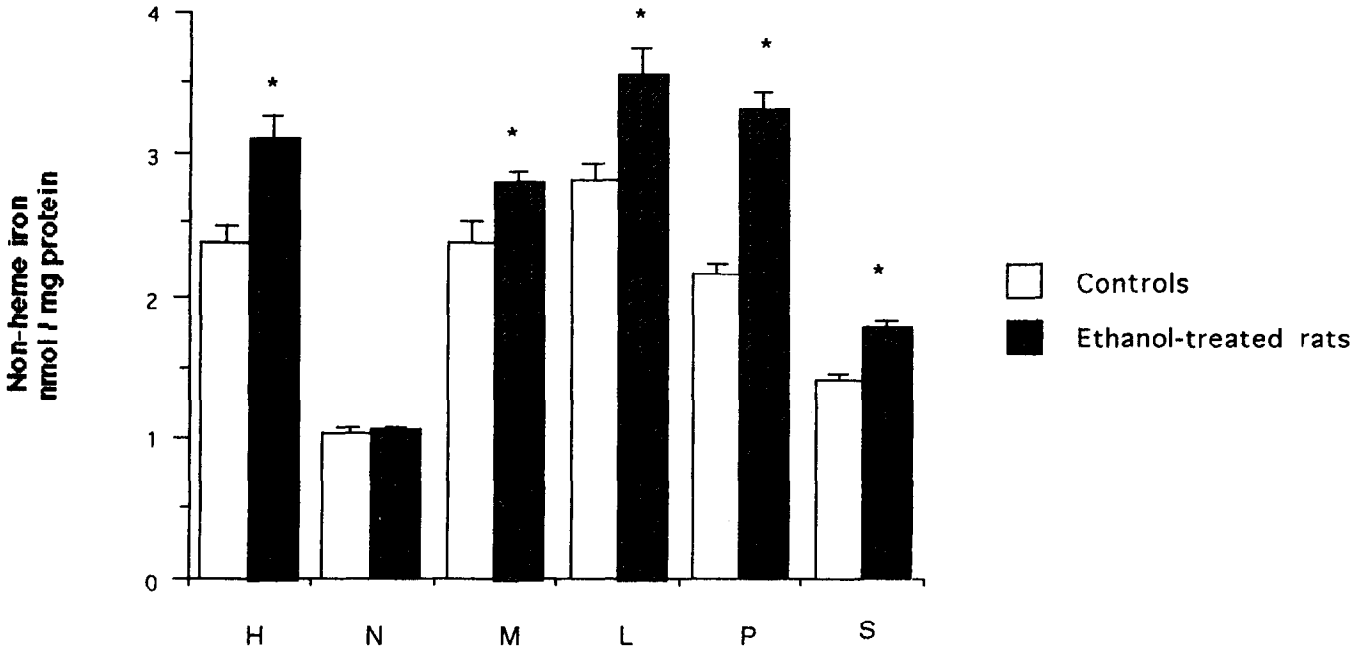

FIG. 1. Effect of chronic ethanol administration on the subcellular distribution of nonheme iron in the cerebellum. Ethanol-treated rats were given 10% ethanol (v/v) as sole drinking fluid for 4 weeks. H, homogenate; N, crude nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosolic fraction. The results are means ± SEM for 10 controls and 10 ethanol-treated rats. *significantly different from the control group ($p < 0.001$).

TABLE 1. Effect of chronic ethanol administration on the total nonheme iron and the low-molecular-weight iron contents of the cytosolic fraction from cerebellum

	Controls	Ethanol-treated rats
Total nonheme iron (nmol/mg protein)	1.39 ± 0.04	1.79 ± 0.04*
Low-molecular-weight iron —nmol/mg protein	0.069 ± 0.003	0.107 ± 0.004*
—percent of cytosolic nonheme iron	4.9 ± 0.4	5.9 ± 0.2*

Ethanol-treated rats were given 10% ethanol (v/v) as sole drinking fluid for 4 weeks. Values are means ± SEM for 10 controls and 10 ethanol-treated rats.
* Significantly different from the control group ($p < 0.001$).

alcohol-treated rats. First, ethanol could alter iron turnover, either by increasing iron uptake in the cerebellum and/or by decreasing iron efflux from the cerebellum. Transferrin and transferrin receptors that are widely expressed in the brain [29] may represent a critical site affected by ethanol. Our previous data [30] have shown that an acute ethanol load increases cerebellar uptake of iron from [^{59}Fe] transferrin. Such an increased uptake may participate, at least partly, in cerebellar iron accumulation following chronic ethanol administration. Another possible mechanism may be represented by an ethanol-induced enhancement of iron translocation (neuronal transport) from other iron-rich brain regions [31]. This disturbance may itself result from an impairment in the GABAergic system, the distribution of iron inside the brain appearing to parallel this system [32], which is affected inside the cerebellum following chronic ethanol administration [33].

The bulk of nonheme iron inside the brain is, like in other tissues, safely sequestered inside iron-storage proteins, mainly represented by ferritin. Only a small fraction of total nonheme iron is able to act as a catalyst in the Haber-Weiss reaction, which generates the highly oxidizing and aggressive ·OH radical from superoxide ($\cdot O_2^-$) and hydrogen peroxide. Halliwell [34] therefore emphasized the importance of examining the availability and distribution of "catalytic" metal ions in explaining oxidative damage to brain cells. Our results indicate that LMWC-iron obtained from the cerebellar cytosolic fraction of control rats represented

TABLE 2. Effects of chronic ethanol administration on trace elements and α-tocopherol contents in rat cerebellum

	Controls	Ethanol-treated rats
Zinc	177 ± 6	204 ± 10*
Copper	88.1 ± 0.6	64.5 ± 5.8*
Selenium	11.0 ± 0.5	7.14 ± 0.6*
α-Tocopherol	31.8 ± 1.0	29.6 ± 0.6*

All results are expressed as nmol/g wet wt. Ethanol-treated rats were given 10% ethanol (v/v) as sole drinking fluid for 4 weeks. Values are means ± SEM for 10 controls and 10 ethanol-treated rats.
* Significantly different from the control group ($p < 0.01$).

TABLE 3. Effects of chronic ethanol administration on glutathione-related cytosolic enzymes in rat cerebellum

	Controls	Ethanol-treated rats
Se-glutathione peroxidase (nmol/min/mg prot.)	32.2 ± 2.6	32.7 ± 2.5†
Total glutathione peroxidase (nmol/min/mg prot.)	51.9 ± 7.4	58.4 ± 7.4†
Glutathione S-transferase (nmol/min/mg prot.)	78.7 ± 9.6	105 ± 6.5*

Ethanol-treated rats were given 10% ethanol (v/v) as sole drinking fluid for 4 weeks. Values are means ± SEM for 6 controls and 6 ethanol-treated rats.
* $p < 0.01$; † n.s.

4.9% of the total nonheme iron content. A similar percentage had previously been reported in rat cortical homogenates [35]. Following chronic ethanol feeding, we observed not only an increased LMWC-Fe content, but also a higher percentage of LMWC-Fe in total cytosolic nonheme iron. This change in LMWC-Fe content could be linked to an increase in iron uptake, Breuer *et al.* [36] having shown in K562 cells that iron acquired from transferrin is maintained in the cytosol as a transit pool of chelatable iron (II). An alternative hypothesis relates this increase to iron mobilization from ferritin or nonferritin sources by reducing agents [37, 38] such as superoxide radicals [39] or NADH [40]. Acetaldehyde resulting, itself, from ethanol oxidation in the brain through catalase, cytochrome P 450 IIE1, or alcohol dehydrogenase may be responsible for the production of NADH when oxidized by aldehyde dehydrogenase [41]. Furthermore, the ethanol-induced changes in oxidoreductive status could modify the rate of resequestration of iron [36]. It may also be suggested that ethanol-induced iron changes in LMWC-Fe are linked to disturbances in the iron regulatory protein, a cytosolic protein modulated by variations in cellular iron levels, which exists as either an iron-responsive element-binding protein in the apo-form or a [4 Fe-4 S] cluster protein that shows aconitase activity [42].

Contrasting with the increase in nonheme iron, a decreased copper content was observed in the cerebellum of

TABLE 4. Effects of chronic ethanol administration on cellular free radical targets in rat cerebellum

	Controls	Ethanol-treated rats
Lipid peroxidation (nmol malondialdehyde/min/g prot.)	112 ± 33	101 ± 36†
Protein carbonyl (nmol/mg prot.)	0.50 ± 0.14	0.56 ± 0.15†
Glutamine synthetase (nmol/min/mg prot.)	0.53 ± 0.04	0.50 ± 0.03†

Ethanol-treated rats were given 10% ethanol (v/v) as sole drinking fluid for 4 weeks. Values are means ± SEM for 7 to 9 rats per group.
† n.s.

the chronic ethanol-fed rats. An inverse relationship between the tissue content of iron and copper has previously been observed in the liver [43] and also been found in the *substantia nigra* during Parkinson's disease [44]. The changes in the copper level may, therefore, be related to the increase in the cerebellar iron content.

Selenium levels were markedly decreased in the cerebellum of the ethanol-treated rats. However, this decrease did not affect the selenium-dependent GPx activity. An absence of correlation between selenium content and GPx activity is not totally unexpected, the selenium present in GPx representing only one fifth of total selenium in brain tissue [45]. The remaining selenium is present in other selenoproteins that could play a role in selenium transport [46] and in antioxidant defense [47]. Ethanol-induced selenium decrease may, therefore, affect cerebellar selenoproteins involved in the antioxidant defense, but different from GPx.

Chronic ethanol feeding also elicits a decrease in cerebellar α-tocopherol, which is the major lipid-solube antioxidant and which prevents oxidative attack on membrane-associated lipids [48].

Whereas the presently reported disturbances in total nonheme iron, LMWC-Fe, copper, selenium and α-tocopherol are similar to those that we previously reported after an acute ethanol load [10, 13], strikingly different changes were found concerning zinc and GST.

Contrasting with the decrease in zinc content elicited by an acute ethanol load [13], we presently observed a significant increase in cerebellar zinc in chronic ethanol-fed rats. Zinc plays a role in the antioxidant defense by competing with redox-active metals (e.g. iron and copper) for membrane binding sites. Zinc may also suppress lipid peroxidation through induction of metallothionein [49]. The synthesis of this protein is often induced in response to oxidative stress and, therefore, represents part of the cellular antioxidant mechanisms [50]. Thus, zinc appears a key element in modulating antioxidant interactions, and its increase presently reported in chronic ethanol-fed rats may represent an adaptative response enhancing the antioxidant defense.

The marked increase in GST activity in the cerebellum of chronic ethanol-treated rats may also represent an adaptative response alleviating the toxic effects of ethanol. Indeed, no disturbances in this activity were observed in the cerebellum of rats 4 hr after an acute ethanol load (50 mmol/kg, i.p.) (results not shown). GSTs are abundant in astrocytes and oligodendrocytes within the brain, and constitute a group of multifunctional proteins that are involved in the biotransformation of neurotoxic compounds. Moreover, a GPx activity that does not catalyze the reduction of H_2O_2, but which can utilize lipid hydroperoxides as substrates is associated with certain forms of GSTs. An induction of GST has been reported in the brain of selenium- and vitamin E-deficient rats, an induction which has been related to the increase in lipid peroxidation products generated during dietary antioxidant deficiency [51].

Contrasting with the changes in the cerebellar pro- and antioxidants, no disturbances were observed in cerebellar lipid peroxidation and protein carbonyl content in rats fed ethanol for 4 weeks. Overt indices of oxidative lipid or protein damage are, thus, lacking at this stage of ethanol administration. This does not prove that free radical mechanisms similar to those previously reported following an acute ethanol load [10, 13] were totally absent in the present model of moderate chronic ethanol consumption. It appears more likely that the increase in some antioxidants (zinc and GST, as well as other putative elements not studied herein) is able to avoid the occurrence of an oxidative stress that would result from the imbalance between the increase in LMWC-Fe, a pro-oxidant factor, and the decrease in prominent antioxidants, such as α-tocopherol and selenium.

The adaptative increase in some antioxidants is, however, not sufficient to prevent a decrease in the cerebellar activity of glutamine synthetase when the ethanol feeding period is extended to 8 weeks. In the brain, this enzyme appears specially sensitive to inactivation by oxygen radicals [52] and plays a pivotal metabolic role [53]. An increase in superoxide production has been previously reported [54] in brain submitochondrial particles isolated from rats submitted to the same model of ethanol administration. A further source of enhanced radical production in the central nervous system following chronic ethanol administration is the ethanol-inducible cytochrome P450 IIE1 [55, 56]. The decrease in the glutamine synthetase activity presently reported following mild long-term conditions of ethanol administration may represent a critical factor in ethanol-induced neurotoxicity. It might, indeed, result in glutamate accumulation leading to increased calcium flux through receptor-gated and/or voltage-sensitive calcium channels, contributing to free radical-induced neuronal toxicity [41]. Such an increase in the cerebellar glutamate content has previously been reported after short-term ethanol administration [57].

Whereas some previous reports had shown that chronic administration of large amounts of ethanol producing sustained elevated blood ethanol levels resulted in overt indices of oxidative stress in rat brain [41, 55, 58], our present findings show that disturbances in some pro- and antioxidants are present even following chronic administration of ethanol representing only 18% of ingested calories. These changes may result in an accumulation of lipofuscin pigment, as previously described in the cerebellum of alcohol-treated rats [59].

Most of the ethanol-related alterations are similar to those described in diseases related to age [60]. It can, therefore, be suggested that an oxidative stress has a pathogenetic role in brain damage related to chronic alcoholism as in some neurologic degenerative diseases.

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