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European Journal of Pharmacology 459 (2003) 171–178
www.elsevier.com/locate/ejphar

Excitatory and inhibitory amino acid changes during repeated episodes of ethanol withdrawal: an in vivo microdialysis study

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Received 30 September 2002; received in revised form 22 November 2002; accepted 26 November 2002

Abstract

Changes in amino acid levels in the hippocampus during repeated ethanol withdrawal were studied. Wistar rats were made ethanol-dependent by 4-week vapour inhalation. After this first cycle of chronic ethanol treatment, rats underwent repeated and alternate cycles of 24 h of withdrawal followed by 1 week of chronic ethanol treatment. The microdialysis technique was used together with high-performance liquid chromatography and electrochemical detection to separate and quantify different amino acids such as aspartate, glutamate, arginine, taurine, alanine and γ-aminobutyric acid. During the first cycle of ethanol withdrawal, increases in glutamate, taurine and alanine levels were observed. During the third withdrawal period, further increases were detected in aspartate, glutamate, taurine and alanine in the comparison with levels in the control group. However, the arginine level during the third withdrawal period decreased when compared to that after the first withdrawal period. The results of this study demonstrate that excitatory amino acid levels increased with subsequent withdrawal periods.

Keywords: Ethanol withdrawal, repeated; Amino acid; Microdialysis; Hippocampus

1. Introduction

Long-term exposure to ethanol leads to an imbalance in different excitatory and inhibitory amino acids (Faingold et al., 1998). When ethanol consumption is reduced or completely stopped, these imbalances in different amino acids and neurotransmitters are behaviourally expressed in the form of ethanol withdrawal and its symptoms (Jaffe, 1990).

Excitatory amino acids, particularly glutamate, have been implicated in ethanol withdrawal (Rossetti and Carboni, 1995; Dahchour and De Witte, 1996; Tsai et al., 1998) and may be responsible for the seizures, anxiety, hyperexcitability and even neuronal death due to excitotoxicity (Chandler et al., 1993; Grant et al., 1990; Lovinger, 1993; Nagy and László, 2002) observed during ethanol withdrawal. Regarding inhibitory amino acids, especially γ-aminobutyric acid (GABA), it has been suggested that both alterations in GABA and its receptors occurring during chronic ethanol treatment may be also responsible for the behavioural manifestations arising with ethanol withdrawal (Littleton, 1998; Romach and Sellers, 1991).

If both glutamate and GABA and their receptors are responsible, at least partly, for ethanol withdrawal symptoms, then the mechanism by which ethanol exerts its effects on two amino acids is well characterised, i.e. an inhibition of NMDA receptor function (Lovinger et al., 1989) and an enhancement of $GABA_A$ receptor function (Ticku and Burch, 1980; for review, see Mihic, 1999). Chronic ethanol consumption leads to an up-regulation of NMDA receptors (Grant et al., 1990; Hoffman and Tabakoff, 1994) and a down-regulation of $GABA_A$ receptor-mediated function (Grant and Lovinger, 1995; N'Gouemo et al., 1996).

The combination of these two mechanisms is suggested to be responsible for the increase in seizure susceptibility during ethanol withdrawal (Faingold et al., 2000; Kang et al., 1996; Mhatre and Ticku, 1992). Furthermore, the hypofunction of $GABA_A$ receptors and the enhanced function of NMDA receptors may be exacerbated by

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doi:10.1016/S0014-2999(02)02851-0

repeated withdrawal (Gonzalez et al., 2001). Moreover, animal studies have shown that alternate multiple periods of ethanol intoxication and withdrawal cause an increased severity of seizures (Becker and Hale, 1993, Becker et al., 1997).

Since multiple and repeated periods of chronic ethanol consumption and withdrawal often occur in alcohol abusers, study, in an animal model, of the neurochemical changes in different amino acids following chronic ethanol treatment interrupted by repeated ethanol withdrawal episodes may have be of clinical importance and be relevant for the development of treatment strategies.

The purpose of the present study was to assess the effects of repeated ethanol withdrawal on extracellular changes in different amino acids such as aspartate, glutamate, arginine, taurine, alanine and GABA within the hippocampus of freely moving male rats. The hippocampus would be expected to play an important role during detoxification since this brain region has been implicated in anxiety and seizures (Gray, 1982), which are typically associated with detoxification. Furthermore, it is known that the hippocampus is involved in learning, memory and control of behaviour (Douglas, 1967; Traub and Miles, 1991) and, therefore, may be involved in associative processes occurring between the effect of ethanol withdrawal and environmental factors.

2. Material and methods

2.1. Experimental design

Fig. 1A shows that the duration of the first cycle of chronic ethanol treatment was 4 weeks, while that of the second and the third cycles was 1 week. Each withdrawal period lasted 24 h, starting at 8:00 p.m. The microdialysis experiment was performed during 24 h (Control) or during the first 24 h of the withdrawal period (W1) (for the second group) and during the third withdrawal period of 24 h (W3) (for the third group).

2.2. Chronic ethanol treatment and surgery

Male Wistar rats (300–350 g) were individually housed in plastic cages and kept in a temperature- and light-controlled environment (light/darkness cycle: 12-h light/12-h dark cycle) made of an isolated plastic chamber ($160 \times 60 \times 60$ cm). A mixture of alcohol and air was pulsed into the chamber via a mixing system, allowing the quantity of alcohol to be increased every 2 days during the whole experimental procedure (Le Bourhis, 1975) for 4 weeks. The control group was not exposed to ethanol vapour but only placed in air vapour chambers under conditions similar to those of the ethanol group.

During the third week, the rats were removed from the chamber for surgery, which was performed under anaesthesia with chloral hydrate (400 mg/kg, i.p.). Using standard stereotaxic techniques, a guide cannula (20-gauge stainless steel; Small Parts, Miami, FL) was implanted 1 mm above the hippocampus (A/P −4.3 mm, M/L 4.0 mm, D/V −3.0 mm) (Fig. 1B) (Paxinos and Watson, 1982) and secured to the skull with three steel screws and cranioplastic cement.

2.3. Microdialysis procedure

After the fourth week of chronic ethanol treatment, or air inhalation for the control group, a concentric dialysis probe was inserted through the guide cannula into the hippocampus region. The dialysis membrane (0.20 mm ID, molecular weight cut-off 18,000 Da) extended 3 mm beyond the tip of the cannula and was continuously perfused with Ringer's solution (145 mM NaCl, 4 mM KCl, 1.3 mM $CaCl_2$; pH 7.2) by a microinfusion pump (Infusion syringe pump 22, Harvard apparatus) at 1 μl/min. Microdialysis samples were collected every 20 min for 24 h.

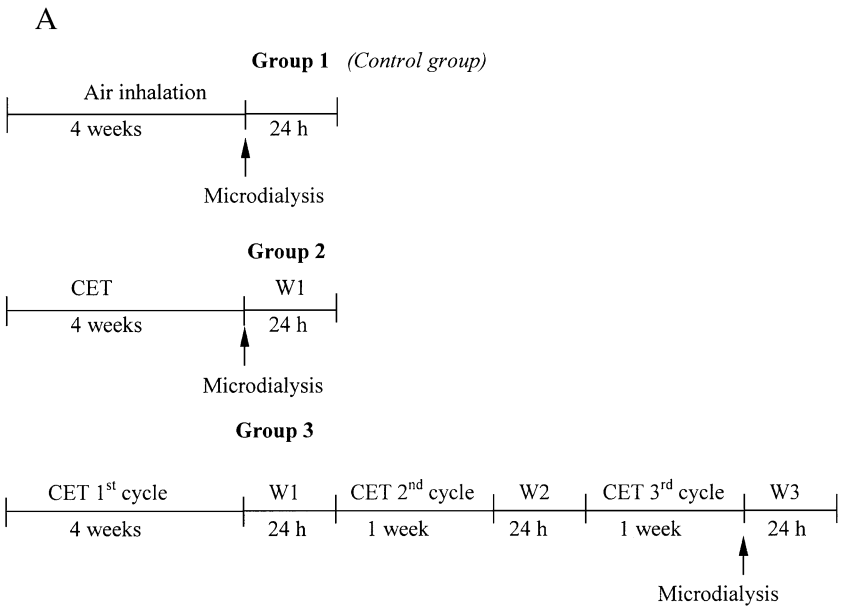
Microdialysis experiments were carried out during the 24-h withdrawal periods. The first group (control group) was placed in the air chamber for 4 weeks and then the microdialysis experiment was performed. The second group of animals was chronically exposed to alcohol for 4 weeks. After that, the microdialysis experiment was performed during the first 24 h of ethanol withdrawal.

The third group was also withdrawn from alcohol for 24 h (W1) but without performing the microdialysis experiment. Then, the same group was placed back into the chamber for a fifth week of chronic ethanol treatment before another 24 h withdrawal (W2) with no microdialysis; then, back into the chamber for a sixth week of chronic ethanol treatment before the third 24-h withdrawal period. During this last withdrawal period (W3), the microdialysis experiment was performed.

These experiments were approved by the Belgian governmental agency under the authorisation number LA 1220028 as well as the European Communities Council Directive concerning the Use of Laboratory Animals.

2.4. Electrochemical detection

The concentration of aspartate, glutamate, arginine, taurine, alanine and GABA in each of the microdialysate samples was analysed by high-performance liquid chromatography (HPLC) with electrochemical detection and *O*-phthaldialdehyde/β-mercaptoethanol precolumn derivatisation (Donzanti and Yamamoto, 1988). *O*-phthaldialdehyde (27 mg) was dissolved in 1 ml methanol HPLC-grade to which 10 μl β-mercaptoethanol was added. This solution was diluted with 9 ml of 0.1 M sodium tetraborate buffer, pH 9.3, and stored at 4 °C. The working solution was prepared each day, 24 h before use, by diluting 1 ml of the above solution in 3 ml of 0.1 M sodium tetraborate. The derivatisation procedure entailed mixing the dialysate



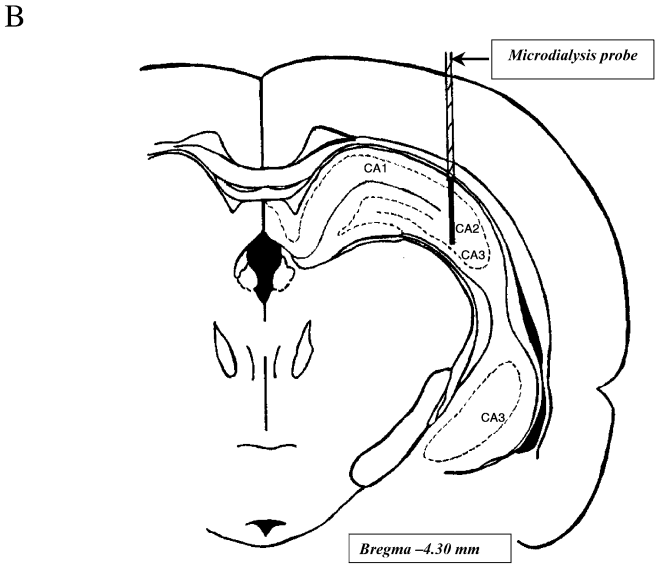


Fig. 1. (A) The experimental design. CET = chronic ethanol treatment. W1, W2 and W3: first, second and third withdrawal periods, respectively. (B) The location of the microdialysate probe. A guide cannula was placed in the hippocampus (anterioposterior −4.3 mm, lateral 4.0 mm, ventral −3.0 mm from the bregma). The microdialysate probe was inserted into the cannula at the beginning of the microdialysis experiment.

(15 μl) and the internal standard (10 μl of homoserine 5×10^{-6} M) with 10 μl *O*-phthaldialdehyde/β-mercaptoethanol for 2 min in complete darkness, and 20 μl of this mixture was injected into HPLC system. This system consisted of a LDC Consta Metric 3200 pump delivering 1 ml/min of the mobile phase at a pressure of 5300 psi. Separation of amino acids was achieved on a reversed-phase column (125 × 3 mm, ODS Hypersil 3 μm) (VDS Optilab) and detected coulometrically (ESAII, Bedford, MA) using three electrodes: a guard (500 mV), preoxidation (180 mV) and working (425 mV) electrode (Analytical cell ESA Model 5011). The mobile phase used (0.1 M Na_2HPO_4, 0.134 mM EDTA, 27% of methanol HPLC-grade, 73% Milli-Q H_2O, pH 6.4) was filtered through a 0.2-μm cellulose nitrate filter (Gelman Sciences, Ann Arbor, MI) and degassed under vacuum before being used in the HPLC system.

Table 1
Blood alcohol levels for the 24-h ethanol withdrawal period of a separate group of nine rats

Time after withdrawal (h)	Blood alcohol level (g/l)
0	2.511 ± 0.075
12	0.153 ± 0.020
24	0.016 ± 0. 002

Blood alcohol levels are presented as the mean concentration (g/l) ± S.E.M.

The position and height of peaks of the endogenous components were compared with those of a standard solution containing glutamate, aspartate, arginine, taurine, alanine and GABA that was prepared in a solution of Milli-Q water and HPLC-grade methanol (50:50 v/v). The working solution was prepared each day by diluting the stock solution to 10^{-6} M in Ringer's solution and 20-μl samples of this solution were injected and quantified. All reagents used were of analytical grade from Sigma (St. Louis, MO). An HPLC autosampler (Model 465, Kontron Instruments, Milan, Italy) was used for sample injection, and the heights and areas of the peaks were quantified by a PC Integration Pack (Kontron Instruments).

2.5. Histology and statistical analysis

Upon completion of the three cycles of alcohol intoxication and withdrawal, the rats were killed and their brains were fixed with 10% formalin. Coronal sections through the extent of the cannula tracks were cut (100 μm) with a vibratome (Polaron H 1200, Biorad, Cambridge, MA) and stained with 0.5% cresyl violet. Dialysis probe

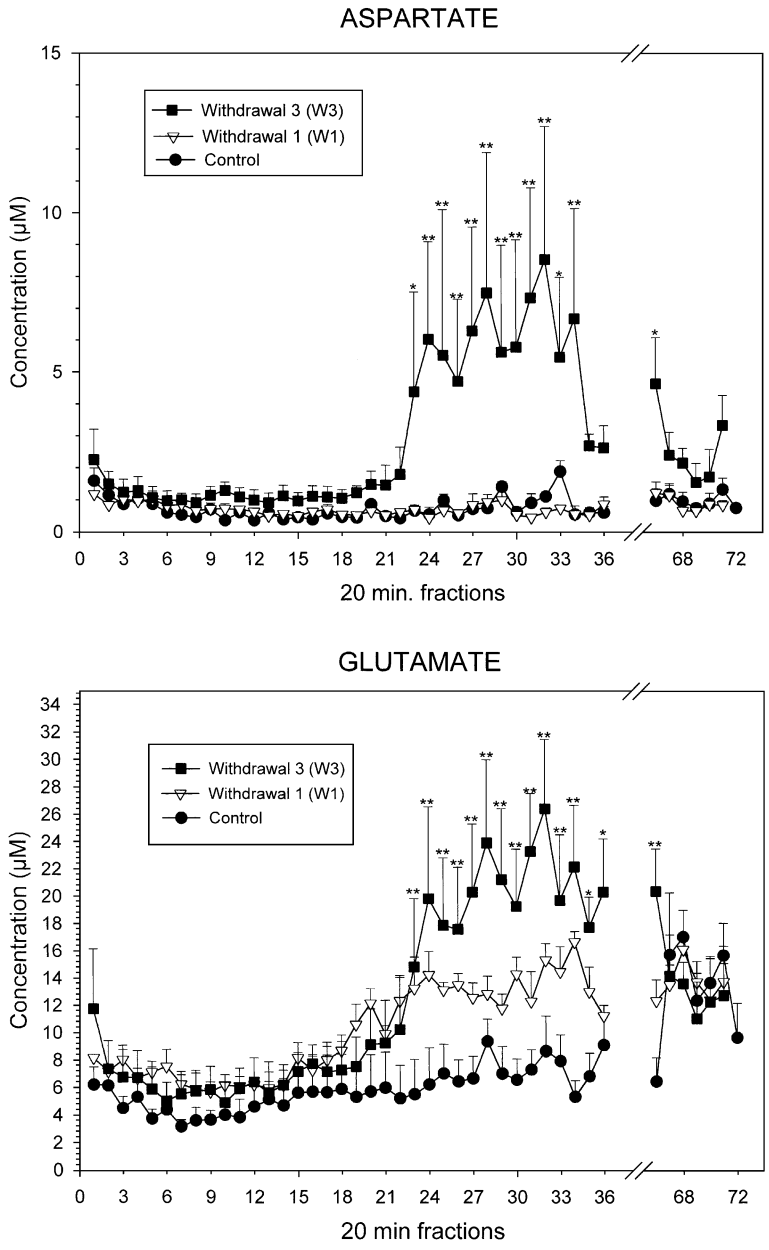


Fig. 2. The effects of repeated withdrawal on aspartate (1) glutamate (2), arginine (3), taurine (4) and alanine (5) microdialysate levels as assayed during the first (W1) ($n=8$) and the third (W3) ($n=6$), for each amino acid, period of ethanol withdrawal by fractions of 20 min, respectively. Data are represented as mean concentration (μM) ± S.E.M. Statistical significance is represented by $*P<0.05$, $**P<0.01$ compared to the relevant control group ($n=8$) not exposed to chronic ethanol treatment.

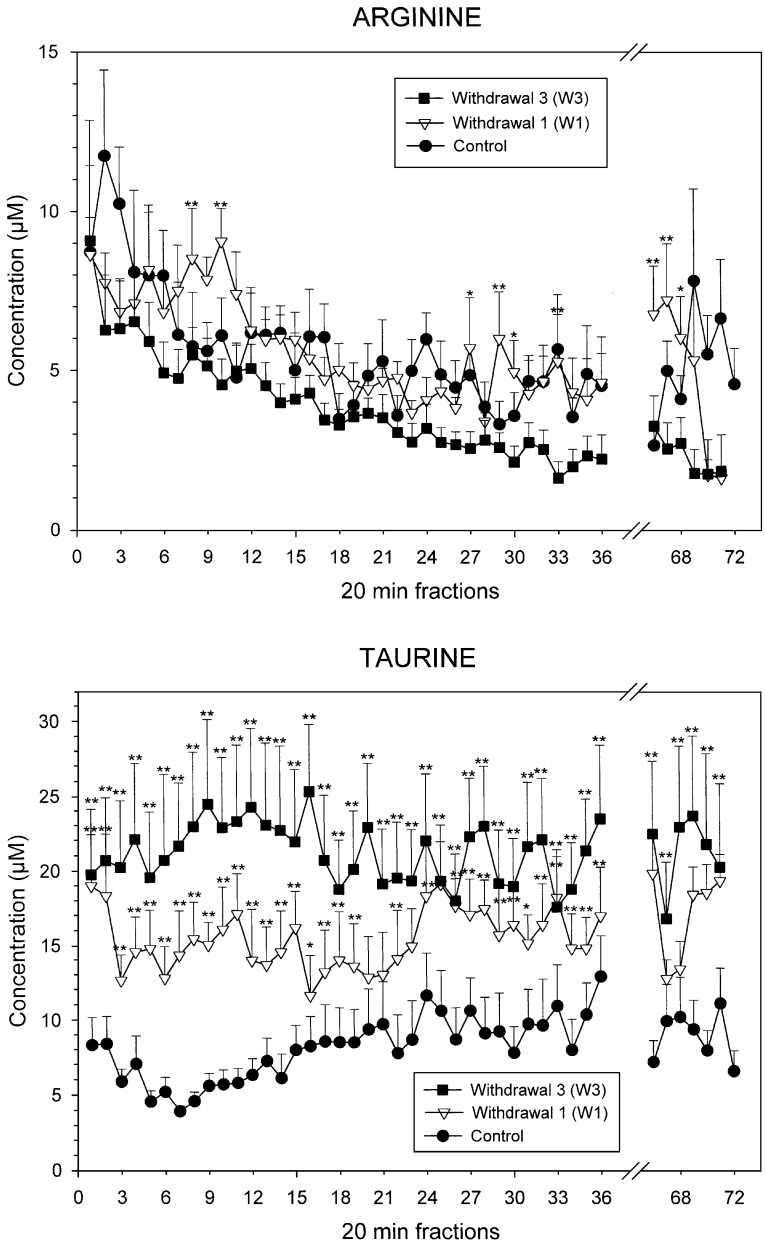


Fig. 2 (*continued*).

placement was localised according to the atlas of Paxinos and Watson (1982).

Data were analysed by analysis of variance with repeated measures (ANOVA), followed by the least-significant difference test of multiple comparison (Fisher's least significant differences (LSD) protected *t*-test) to evaluate group and time differences (GB-STAT, Dynamic Microsystems, Silver Spring, MD).

2.6. Blood alcohol level during ethanol withdrawal

Blood alcohol level was measured during 24 h of ethanol withdrawal (Table 1) in a separate group of nine rats. Blood samples were taken from the tail three times, i.e. at the beginning of ethanol withdrawal, 12 h after ethanol withdrawal (*t*12) and 24 h after ethanol withdrawal (*t*24). The blood ethanol levels were assayed by the alcohol dehydrogenase method (Boehringer Mannheim, Germany).

3. Results

3.1. Effect of repeated withdrawal on extracellular concentration of amino acids aspartate, glutamate, arginine, taurine, alanine and GABA

The data shown in Fig. 2 refer to the effects of repeated withdrawal on the amino acids aspartate, glutamate,

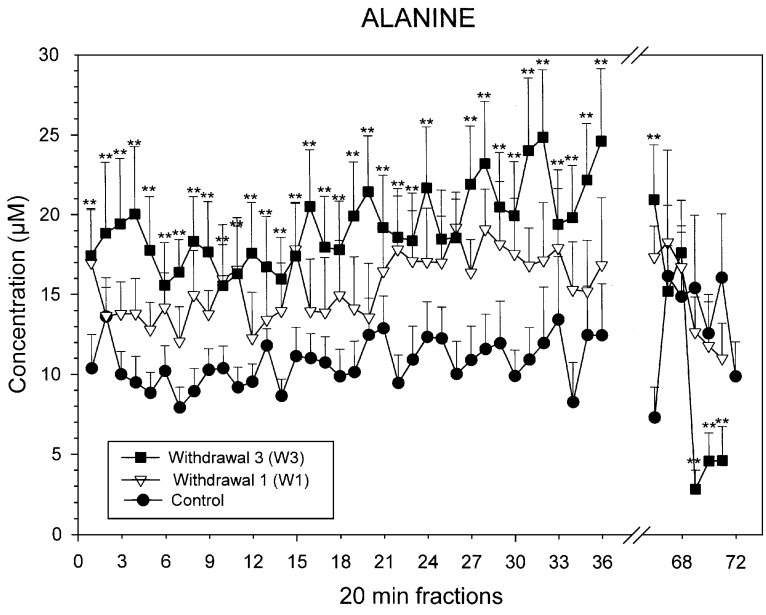


Fig. 2 (*continued*).

arginine, taurine and alanine, as assayed during the first and the third 24-h cycles of ethanol withdrawal. These data were compared with those of the control group that inhaled only air during the same period. Aspartate levels (Fig. 2.1) were significantly increased during the third repeated ethanol withdrawal period when compared with those in the control group [$F(1,492)=6.450$; $P=0.029$]. Analysis of variance showed a significant difference in time [$F(41,503)=3.115$; $P<0.0001$] and in the interaction between time and groups [$F(41,503)=2.549$; $P<0.0001$]. However, ANOVA did not reveal any significant changes between aspartate levels during the first ethanol withdrawal period and those of the control group. [$F(1,615)=0.198$; $P=0.663$].

Fig. 2.2 shows a subsequent increase in glutamate levels during repeated cycles of ethanol withdrawal in comparison with levels in the control group. Analysis of variance showed that glutamate levels were significantly increased during the third withdrawal period compared with those of the control group [$F(1,574)=5.26$; $P=0.040$]. There were also significant differences in time [$F(41,587)=5.11$; $P<0.0001$] and in interaction between time and groups [$F(41,587)=2.149$; $P<0.0001$]. However, even though glutamate levels were significantly increased in time [$F(41,671)=10.352$; $P<0.0001$], ANOVA did not show any significant differences between glutamate levels during the first ethanol withdrawal period and those of the control group [$F(1,556)=1.637$; $P=0.22$].

There were no significant differences in arginine levels (Fig. 2.3) during the first or the third ethanol withdrawal period compared with those of the control group. However, the arginine levels during the third ethanol withdrawal period were significantly lower [$F(1,533)=5.156$; $P=0.044$] than the arginine levels during the first ethanol withdrawal.

Taurine levels (Fig. 2.4) were high in the first [$F(1,656)=10.663$; $P=0.006$] and the third [$F(1,533)=10.944$; $P=0.007$] ethanol withdrawal periods when compared with those of the control group.

Alanine levels (Fig. 2.5) were higher in the third ethanol withdrawal period than in the control group [$F(1,574)=6.05$; $P=0.03$]. However, ANOVA did not show any significance between alanine levels in the first ethanol withdrawal and those of the control group [$F(1,656)=2.87$; $P=0.112$]. Although the level of GABA was very low during the third withdrawal period, analysis of variance did not show any significant differences in GABA levels (figure not shown) during the first ethanol withdrawal period or during the third ethanol withdrawal period in comparison with those of the control group.

4. Discussion

We reported previously that there was a dramatic increase in glutamate levels in the microdialysate from the nucleus accumbens during the first ethanol withdrawal period (Dahchour and De Witte, 1996). These findings have now been extended in order to assess whether or not repeated and alternate cycles of chronic ethanol treatment and withdrawal alter the amino acid microdialysate content, particularly that of aspartate, glutamate, arginine, taurine, alanine and GABA, in the hippocampus of male Wistar rats.

There have been many previous behavioural investigations of the effects of repeated ethanol withdrawal on the severity of withdrawal episodes and hypermotility in rats (Poldrugo and Snead, 1984; Veatch and Gonzalez, 1996; Dahchour and De Witte, 1999) and mice (Becker and

Hale, 1993; Becker et al., 1997). These studies have reported an increase in seizure scores (Becker et al., 1997), seizure sensitivity (McCown and Breese, 1990) and electrographic activity (Veatch and Gonzalez, 1996). Whether these behavioural processes reflect amino acid neurotransmitter changes, particularly glutamate and aspartate, would be relevant to advancing of our understanding of the basic neurochemical modifications underlying ethanol withdrawal symptoms, as well as to developing new strategies for the achievement of detoxification period and abstinence.

The increased glutamate levels during the first cycle of ethanol withdrawal (Fig. 2.2), when compared to its baseline values, supports our previous studies (Dahchour and De Witte, 1996; Dahchour et al., 1998) and, interestingly, this increase in glutamate levels was paralleled by an increase in aspartate level during the third withdrawal episode (Fig. 2.1). It is noteworthy that during the third cycle of ethanol withdrawal, the increase in both glutamate and aspartate levels was much higher than during the first episode of withdrawal.

Such occurrences could be explained by the fact that the initial increased release of glutamate during the first cycle of withdrawal could have caused neuronal sensitisation to glutamate receptor activation, possibly by increasing Ca^{2+} entry into neurones through the glutamate controlled voltage sensitive channels. The increase in intracellular Ca^{2+} concentration would activate a number of processes that, in turn, promote the release of glutamate by a feedback mechanism. Examples of these processes, include the activation of the enzyme nitric oxide synthase (NOS), which is Ca^{2+}/calmodulin dependent (activated by Ca^{2+}), and the formation of nitric oxide (NO), which diffuses and activates in turn the release of glutamate via guanylate cyclase and cGMP (Grathwaite, 1991; Grathwaite et al., 1989).

Interestingly, the taurine level (Fig. 2.4) was significantly increased during the third withdrawal episode when the levels of the excitatory amino acids glutamate and aspartate were dramatically increased. Taurine is an inhibitory amino acid known to enhance Cl^{-} conductance (Oja et al., 1990), attenuate Ca^{2+} influx and antagonize depolarization-evoked Ca^{2+} efflux in the brain (Kontro and Oja, 1988), and to protect neurones from excitotoxicity induced by excitatory amino acids in the hippocampus (French et al., 1986) and cerebellum (Trenkner, 1990). Therefore, the increased taurine level after ethanol withdrawal may have a protective effect against excitotoxicity induced by glutamate.

The arginine levels in the hippocampal microdialysate were significantly reduced in both groups during subsequent withdrawal period (Fig. 2.3). It is known that L-arginine is the precursor of NO, a second messenger that may have important neurochemical roles. The formation of NO is controlled by the enzyme NOS together with the co-factors Ca^{2+} and calmodulin. Chronic ethanol treatment adversely affects the Ca^{2+}-dependent NOS enzyme (Zhang et al., 1998) and may also influence the two other forms of NOS present in the brain, neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS).

It is likely that Ca^{2+} homeostasis will be altered during the initial period of withdrawal (Bredt and Snyder, 1990) which could increase NO production. The decrease in arginine level during the third ethanol withdrawal period may, therefore, represent an adaptation by the brain to prevent the production of NO by the reduction of its substrate.

The role of alanine in ethanol withdrawal is still largely unknown. The baseline levels of this amino acid were consistently increased in all groups with chronic ethanol intoxication and subsequent withdrawal, but not in the control group (Fig. 2.5). This could be functionally related to increased glutamate levels during ethanol withdrawal, as suggested by the fact that alanine may be a precursor of glutamate and could produce an increase in aspartate under hypoxic and normoxic conditions (Griffin et al., 1998). Similarly, supplementation of alanine has been proposed to protect against hepatotoxicity (Maezono et al., 1996). Further investigations would be of interest to assess whether alanine plays a protective role against central nervous system damage subsequent to ethanol withdrawal.

In this study, we show an elevation in extracellular levels of the excitatory amino acids glutamate and aspartate in the hippocampus after repeated ethanol withdrawal. The elevated glutamate release during the first of ethanol withdrawal episode was exacerbated in subsequent withdrawal episodes. This may be relevant for the development of treatment strategies.

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