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Ethanol withdrawal is associated with increased extracellular glutamate in the rat striatum

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Abstract

Extracellular glutamate was measured by microdialysis in the striatum of ethanol-dependent, freely behaving rats following withdrawal from chronic ethanol treatment. Within 12 h from withdrawal, extracellular glutamate rose to 255% of that in control, chronic sucrose-treated rats. Glutamate output remained elevated for the subsequent 12 h and returned to control levels within 36 h from the interruption of the treatment. The changes in glutamate were time-locked to the overt physical signs of withdrawal. In 12-h ethanol-withdrawn rats an ethanol challenge suppressed the withdrawal signs and reduced the extracellular glutamate. The NMDA receptor antagonist, dizocilpine, reduced both the physical signs of withdrawal and glutamate output. In contrast, diazepam reduced the withrawal signs but failed to change the glutamate levels. These findings suggest that the increased extraneuronal glutamate reflects overactivity of excitatory neurotransmission during withdrawal. Furthermore, they provide a biochemical rationale for the use of NMDA receptor antagonists and ethanol itself in the treatment of ethanol withdrawal syndrome.

Keywords: Ethanol withdrawal; NMDA receptor; $GABA_A$ receptor; Microdialysis; Dizocilpine; Diazepam

1. Introduction

The interruption of ethanol intake in ethanol-dependent subjects results in the development of a severe withdrawal syndrome. This syndrome can be observed in both humans and animals and is characterized by a number of neurological signs such as tremulousness, sympathetic hyperactivity, seizures and death (Victor and Adams, 1953; Freund, 1969; Goldstein, 1973; Ritzmann and Tabakoff, 1976; Tabakoff and Rothstein, 1983; Nutt and Glue, 1990; Romack and Sellers, 1991).

Alterations in the excitatory glutamatergic neurotransmission appear to be an important factor in the neurochemical mechanisms associated with the ethanol withdrawal syndrome. Ethanol is an inhibitor of NMDA receptor function (Lovinger et al., 1989; Dildy-Mayfield and Leslie, 1989; Göthert and Fink, 1989; Hoffman et al., 1989; Gonzales and Woodward, 1990). Thus, chronic inhibition of glutamatergic transmission is expected to result in a rebound overactivation of this excitatory system upon withdrawal. In dependent animals, withdrawal from ethanol is associated with an increase in the number of NMDA sites in brain tissue, a presumed neuroadaptive response to chronic blockade of NMDA receptors by ethanol (Gulya et al., 1989; Snell et al., 1993; Sanna et al., 1993). Blockade of NMDA receptors markedly reduces the withdrawal signs, particularly seizures (Grant et al., 1990; Morrisett et al., 1990; Liljequist, 1991; Danysz et al., 1992).

In animals and in cell cultures chronically exposed to ethanol, NMDA-induced neurotoxicity is potentiated following ethanol withdrawal (Davidson et al., 1993; Chandler et al., 1993; Iorio et al., 1993), and ethanol or NMDA receptor antagonists prevent this effect (Lustig et al., 1992; Chandler et al., 1993; Iorio et al., 1993).

These studies suggest that ethanol withdrawal is associated with increased excitatory amino acid neurotransmission. To provide direct evidence that glutamatergic neurotransmission is activated during ethanol

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withdrawal, we measured by microdialysis the extracellular concentrations of glutamate in the striatum of freely behaving rats withdrawn from chronic ethanol treatment. In the same animals, in the dialysis experiment, we evaluated the withdrawal symptomatology to determine the correlation between neurochemical events and behavior. In addition, we studied the effects on extracellular glutamate of two drugs known to reduce the abstinence symptomatology, namely dizocilpine, an NMDA receptor antagonist, and diazepam, an indirect γ-aminobutyric acid (GABA)-mimetic drug.

2. Materials and methods

2.1. Animals and drugs

Male Sprague-Dawley rats (Charles River, Como, Italy) weighing 200–225 g were used. The animals were housed individually and maintained under conditions of constant temperature, humidity and dark/light (12 h/12 h) cycle. Ethanol 95% (Carlo Erba, Milan, Italy) was administered by gavage in 20% solution in saline. Diazepam (Valium, 10 mg/2 ml ampoules, Roche, Basel, Switzerland) was administered i.p. Dizocilpine (MK-801, Merck, Harlow, UK) was admistered i.p. in 1 mg/5 ml solution in saline.

2.2. Ethanol treatment

In our experiments we used an administration regimen known to produce a robust wihdrawal syndrome (Majchrowicz, 1975). On the first day of treatment, food was withdrawn and the animals were given ethanol intragastrically every 6 h for 6 consecutive days (24 administration sessions), starting at 12:00 p.m. of the first day. Following the initial dose of 5 g/kg, successive doses were adjusted to the degree of ethanol intoxication to maintain steady intoxicating levels of plasma ethanol (Majchrowicz, 1975). Briefly, before each administration session the animals were evaluated by the experimenter and a score from neutrality to coma was assigned. When neutrality was scored, rats received the full dose (5 g/kg); when signs of severe intoxication were scored, such as loss of righting reflex or coma, rats received no ethanol. At intermediate intoxicating levels (defined as sedation, ataxia-1, -2 and -3), an intermediate dose of ethanol was administered (4, 3, 2 and 1 g/kg, respectively). Control rats were given isocaloric sucrose (30% w/v in saline) orally in place of ethanol. Once a day, between two administration sessions, the animals received 4 ml intragastrically of a lyophilized milk solution in saline as the sole source of food. On the day of the dialysis experiments the mean weights of ethanol-treated and control rats were 182 ± 11 and 202 ± 8 g (mean ± S.E.M., $n = 13$), respectively.

2.3. Microdialysis

After the 20th ethanol dose, the animals were anesthetized with chloral hydrate and implanted with the dialysis fiber (active length, 8 mm, AN69HF membranes, Hospal, France) transversely through the caudate (Ungerstedt, 1984; Di Chiara, 1990) with coordinates A: 2 mm and V: 5.5 mm from the bregma (Paxinos and Watson, 1986). In ethanol-intoxicated rats a dose of chloral hydrate of 200 mg/kg i.p. was usually sufficient to produce surgical anesthesia, due to the summation of the effect of chloral hydrate and that of ethanol. Animals then received 4 additional doses of ethanol at 6-h intervals, starting 6 h after surgery. Control rats were implanted in the same manner as ethanol-treated rats, under chloral hydrate anesthesia (400 mg/kg i.p.). At 7:00 a.m. of the 7th day, 1 h following the last treatment and 24 h after the fiber implantation, the rats were connected to the perfusion pump and the dialysis probe was perfused with a modified Krebs buffer, pH 7.4 (composition, mM: $CaCl_2$, 1.2; NaCl, 147; KCl, 3; $MgSO_4$, 1.2; KH_2PO_4, 0.4) at a flow rate of 1.8 μl/min. Sample collection began following 1 h stabilization period.

At the end of each experiment, the animals were killed, the brain was removed and coronal sections were cut to confirm the correct placement of the dialysis membrane.

2.4. Amino acid analysis

Amino acids were measured in 20 μl dialysate samples by reverse-phase high pressure liquid chromatography (15 × 0.4 cm o.d., C_{18} 5 μm particle size column, Supelco) coupled to fluorescence detection (excitation wavelength: 318 nm; emission wavelenght: 452 nm, Kontron SFM 25 spectrofluorimeter), following pre-column derivatization with *o*-phthaladialdehyde/2-mercaptoethanol. The mobile phase was phosphate buffer 0.1 M, pH 6.2, containing methanol 28% v/v and the flow rate was 1 ml/min. The column temperature was maintained at 35°C.

2.5. Recovery in vitro

The efficiency of the diffusion of glutamate through the membrane was measured by perfusing the probe dipped in a beaker containing 1 ml of of 100 μM glutamate solution in Krebs buffer at 37°C. The relative recovery was 88 ± 4%.

2.6. Assessment of overt withdrawal signs

Rats were tested for withdrawal signs and assigned a withdrawal score according to a standardized protocol as described by Lal et al. (1988). Overt physical signs of withdrawal were assessed by evaluating spontaneous behaviors (general activity, treading, shakes, jerks and twitches), response to handling (vocalization) and neurological signs (rigidity, tail tremor, caudal tremor, general tremor, catalepsy, bracing posture and convulsions). Each item was evaluated separately by the rater assigning a score from 0 to 3 and the sum of the scores was taken as a measure of the withdrawal syndrome (see Lal et al., 1988, for details).

2.7. Statistical analysis

Neurochemical data were analyzed by analysis of variance (ANOVA) for repeated measures, or by one-way ANOVA followed by the Newman-Keuls test, where appropriate. Behavioral data were analyzed by means of the Mann-Whitney non-parametric test.

3. Results

3.1. Changes in glutamate output during withdrawal

In chronic sucrose-treated rats 2 h following the last administration, the glutamate output was 4.7 ± 0.06 pmol/min (mean $\pm$ S.E.M., $n = 9$). Glutamate levels remained stable for at least 36 h (Fig. 1). In ethanol-intoxicated, non- withdrawn rats, i.e. chronic ethanol-treated animals undergoing continued ethanol administration every 6 h, the glutamate output was not statistically different from that in sucrose-treated rats (3.87 ± 1.35 pmol/min, $n = 5$). As in the controls, no change in glutamate output was observed in ethanol- intoxicated rats during the 36 h observation period. In contrast, ethanol withdrawal produced a gradual increase in the perfusate concentrations of glutamate (Fig. 1, top). Glutamate output reached a maximum 12 h after withdrawal (12.7 ± 1.85 pmol/min, $P < 0.001$ with respect to controls, one-way ANOVA), remained elevated for the 12 h following and returned to near the control values 36 h from withdrawal.

In ethanol-withdrawn rats the score of the withdrawal signs was maximal 12 h following the last ethanol administration, remained elevated for the next 12 h and then gradually returned to the control values within 36 h from the last administration (Fig. 1, bottom).

3.2. Effect of an ethanol challenge

In 12-h-withdrawn rats, an ethanol challenge (5 g/kg p.o.) reduced glutamate output to the control values

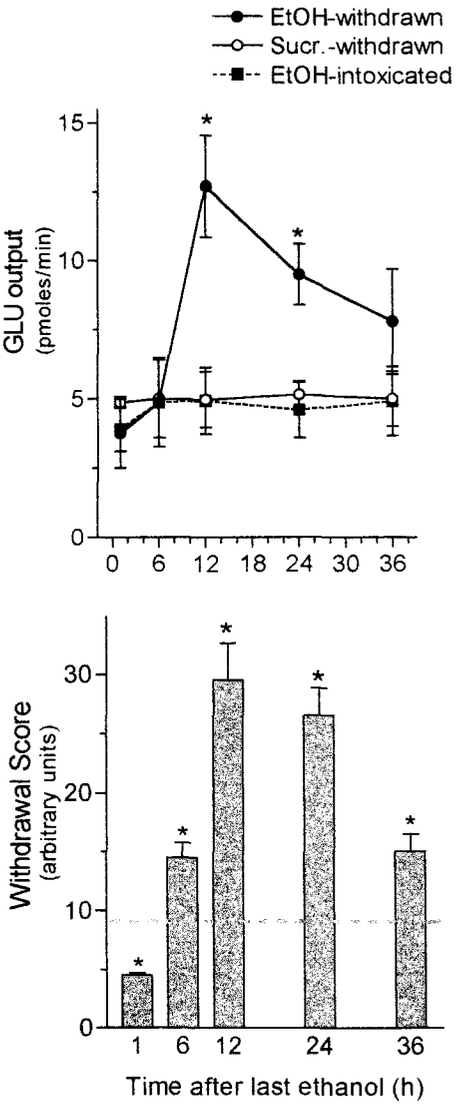

Fig. 1. Changes in the extraneuronal glutamate concentrations in vivo in the striatum of ethanol-dependent, freely behaving rats after cessation of chronic ethanol treatment (top panel) and time course of the withdrawal signs (bottom panel). Rats received ethanol by gavage (20% v/v in saline) every 6 h for 6 days, at a dose (1–5 g/kg) titrated to maintain a steady level of intoxication throughout the treatment. Control rats received an equicaloric sucrose solution (30% w/v in saline). The ethanol-intoxicated group represents chronically treated animals that received ethanol continuosly every 6 h and is shown for comparison. Points represent the average glutamate output from 5–12 rats per group and are means $\pm$ S.E.M. Asterisks represent the statistical significance when compared with control, chronic sucrose-withdrawn rats ($P < 0.05$, one-way ANOVA). Bars represent the mean total score ($\pm$ S.E.M.) of the overt physical signs of withdrawal assessed on 13 behavioral items. The dotted line indicates control values ($^*P < 0.05$ as compared to control values, Mann-Whitney U-test).

within 3 h ($F(1,82) = 3.06$, $P < 0.001$, repeated measures ANOVA). Glutamate output remained near the control levels until about 8 h after the ethanol challenge (Fig. 2, top). Ethanol produced a marked reduction of the withdrawal signs to below control values

within 1 h. The signs gradually increased to the control levels and then returned to pre-administration values (Fig. 2, bottom).

3.3. Effect of dizocilpine and diazepam

The administration of dizocilpine (1 mg/kg i.p.) to 12-h-withdrawn rats reduced glutamate output to about 38% of the control levels ($P < 0.05$, one-way ANOVA) (Fig. 3) within 2 h. Glutamate remained at these reduced levels for about 1 h and then returned to its pre-injection values in about 4 h following the injection. At the time of its peak effect (2 h) dizocilpine significantly reduced the withdrawal score ($P < 0.05$,

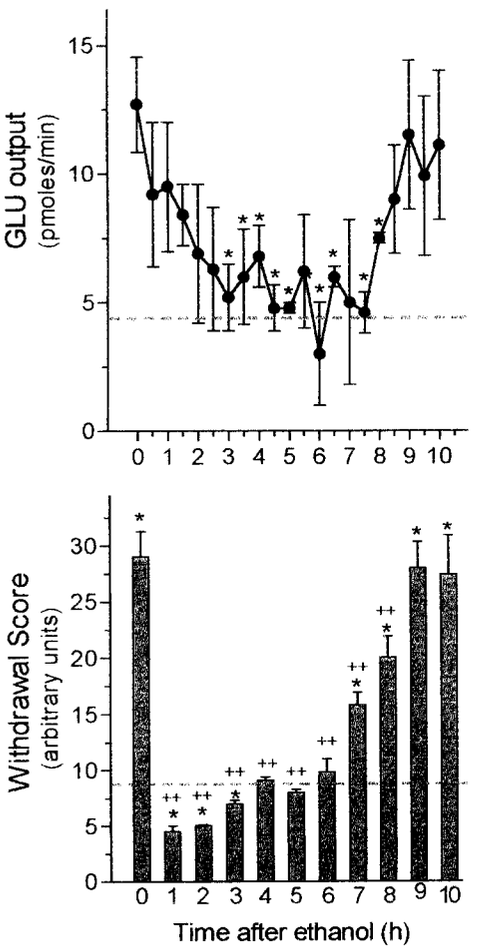

Fig. 2. Effect of an ethanol challenge on striatal extracellular glutamate in vivo (top panel) and on the overt signs of withdrawal (bottom panel) in ethanol-withdrawn rats. Ethanol (5 g/kg) was administered intragastrically 12 h after the interruption of the chronic treatment. Points represents the average perfusate concentrations in 30-min samples and are means ± S.E.M. from 5 animals. Asterisks indicate a significant statistical difference ($P < 0.05$, Newman-Keuls test) with respective time-points in ethanol-withdrawn, saline-treated animals, after ANOVA for repeated measures. Bars represent the mean total score of the overt physical signs of withdrawal assessed on 13 behavioral items and are means ± S.E.M. Dotted lines indicate control values ($^{++}P < 0.05$ as compared to the score in 12-h withdrawn rats; $^{*}P < 0.05$ as compared with control values. Mann-Whitney *U*-test).

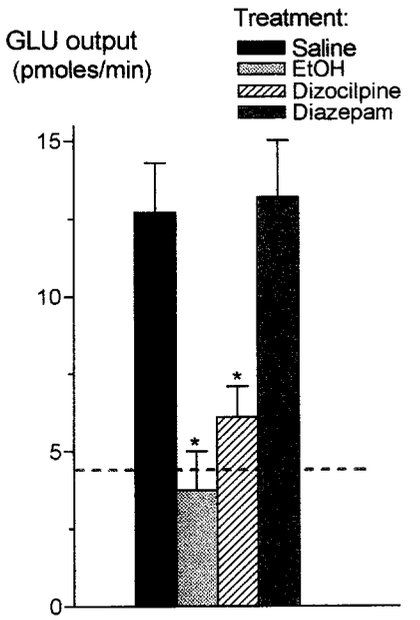

Fig. 3. Effect of ethanol (5 g/kg), dizocilpine (1 mg/kg) or diazepam (5 mg/kg) on striatal glutamate output in 12-h withdrawn rats. Bars represent the average peak glutamate output 1.5–2 h after drug administration and are means ± S.E.M. from 4–5 animals per group. The dotted line indicates control values. $^{*}P < 0.01$, one-way ANOVA.

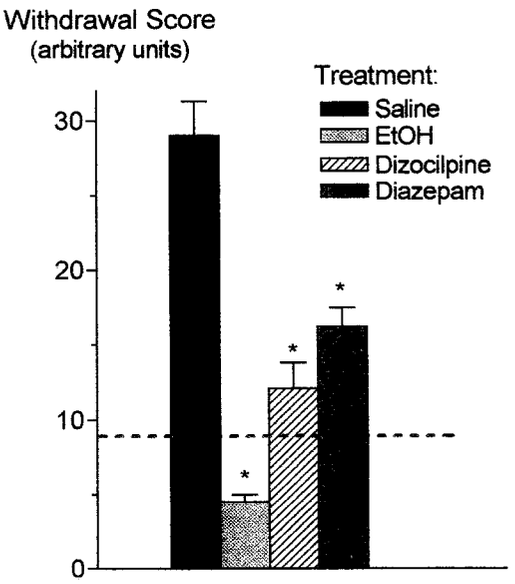

Fig. 4. Effect of ethanol (5 g/kg), dizocilpine (1 mg/kg) or diazepam (5 mg/kg) on the overt withdrawal signs in the rats of the experiment shown in Fig. 3. The withdrawal signs were scored at the same time point of collection of perfusate sample. Bars represent the total withdrawal score as assessed on 13 behavioral items and are means ± S.E.M. The dotted line indicates control values. $^{*}P < 0.05$, Mann-Whitney *U*-test.

Mann-Whitney test) (Fig. 4). Diazepam (5 mg/kg i.p.), in contrast, failed to affect the glutamate output (Fig. 3). However, it reduced the withdrawal score within 30 min from its administration and this reduction lasted for at least 4 h following the injection. Two hours following the administration the withdrawal score was about 46% of the control values ($P < 0.05$, Mann-Whitney test) (Fig. 4).

4. Discussion

The present study was designed to investigate the effects of withdrawal from repeated ethanol treatment on extracellular levels of glutamate in the striatum, a brain area that receives a dense glutamatergic innervation (Fonnum et al., 1981). The interruption of ethanol treatment resulted in a long-lasting increase in the extraneuronal concentrations of glutamate. Increases in extracellular glutamate have also been observed in the cortex and nucleus accumbens (Rossetti, unpublished results) and in the hippocampus (F. Moroni, personal communication) of ethanol-withdrawn rats, so it is likely that the effect observed in the striatum may be an index of an overall increase in excitatory neurotransmission in the brain during ethanol withdrawal.

These results raise the question of whether the increase in glutamate output is behaviorally relevant to the expression of the withdrawal syndrome. The evaluation of withdrawal signs during the dialysis experiments showed a tight, positive correlation between the time courses of the withdrawal symptomatology and the extracellular glutamate (Fig. 5). Although our results do not provide direct evidence for a causal relationship between increased glutamate levels and withdrawal signs, the potentiation of the withdrawal symptomatology by systemic (Grant et al., 1990; Morrisett et al., 1990) or i.c.v. injections of the glutamate receptor agonist, NMDA, in ethanol-dependent rats (Morrisett et al., 1990; Danysz et al., 1992; Sanna et al., 1993) and the prevention of this effect by ethanol or dizocilpine (Grant et al., 1990; Danysz et al., 1992) strongly suggest a functional role for the increased extracellular glutamate in ethanol withdrawal.

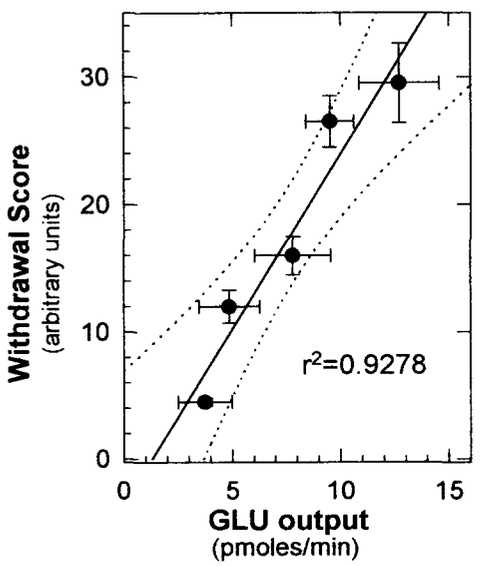

Fig. 5. Linear correlation between behavioral symptomatology and striatal glutamate output. Points are data from the experiment in Fig. 1. The dotted lines represent 95% confidence limits. The slope was significantly different from zero ($P = 0.0081$).

The time courses of the behavioral and the glutamate changes induced by ethanol challenge in 12-h-withdrawn rats had similar time spans; however, the pattern of changes was different. In fact, the ethanol challenge reduced the gross behavioral score more rapidly than the extracellular glutamate. This reduction likely reflects the multiple actions of ethanol (see Deitrich et al., 1989, for review). Beside inhibiting NMDA-mediated glutamatergic transmission ethanol has sedative actions and possesses anesthetic properties. In addition, ethanol has effects on the peripheral nervous system, on muscular tissues, on the vasculature and other organs. More significant in this respect is the sedative and anesthetic effect of ethanol at the dose used. Indeed the data in Fig. 2 show that ethanol initially not only completely suppressed the withdrawal signs but also brought the behavioral score below control values, a result indicating that sedative effects are predominant. The slower decrease in glutamate levels, as compared with the behavioral score, may indicate that glutamate is not involved in the sedative actions of ethanol. Also, more simply, slow equilibration of the concentrations of the amino acid between the outside and the inside of the dialysis membrane may contribute to this discrepancy.

Diazepam was used in our study since a reduced activity of inhibitory GABAergic neurotransmission has been proposed as a neurochemical basis for the excitatory symptomatology of ethanol withdrawal (Hunt, 1983; Allan and Harris, 1987). Although the evidence for changes in $GABA_A$ receptor-mediated function in ethanol withdrawal is controversial (Ticku and Burch, 1980; Liljequist, 1991; Allan and Harris, 1987; Morrow et al., 1988; Buck and Harris, 1990; Sanna et al., 1993), drugs known to enhance GABAergic transmission, such as benzodiazepines and barbiturates, prevent or reverse the ethanol withdrawal syndrome (Goldstein, 1973; Cooper et al., 1979; Frye et al., 1983) whereas drugs that inhibit $GABA_A$ receptor-mediated function exacerbate the signs of withdrawal (Goldstein, 1973; Cooper et al., 1979). Thus diazepam produced the expected reduction of the withdrawal score. However, the failure of diazepam to affect glutamate output at doses that markedly reduce the physical signs of with-

drawal suggests that $GABA_A$ receptors do not modulate glutamate neurotransmission. As for the initial effect of the ethanol challenge, the reduction of the withdrawal signs by diazepam may be ascribed to a general sedative effect. It is possible that benzodiazepines activate inhibitory circuits downstream of the glutamatergic system to reduce the signs of withdrawal. This finding further supports the view that changes in glutamatergic transmission rather than in GABAergic transmission have a primary role in the neurochemical changes associated with ethanol withdrawal (Gulya et al., 1989; Grant et al., 1990; Snell et al., 1993; Sanna et al., 1993; Trevisan et al., 1994).

The reduction of glutamate output by dizocilpine shows that NMDA receptors are involved in the elevation of glutamate output associated with ethanol withdrawal. Considering the antagonist properties of ethanol on NMDA receptors (see the Introduction for references), the reduction in extraneuronal glutamate by the ethanol challenge could be interpreted as a response to NMDA receptor blockade (Carboni et al., 1993). Further support for the role of NMDA receptors in the elevation of extracellular glutamate is provided by the striking similarity between the time courses of extracellular glutamate found in this study and the changes in NMDA receptor density following ethanol withdrawal reported by Gulya et al. (1989). In addition, preliminary evidence from our laboratory shows that the focal application of NMDA in the striatum of ethanol-withdrawn rats induces a potentiation in glutamate output as compared to controls (Rossetti and Carboni, in preparation).

On the other hand, recent findings raise the possibility that the increase in glutamate observed in ethanol-dependent rats might be the result of neurotoxic processes. Increases in extracellular glutamate have been observed in the rat thalamus in an experimental model of thiamine deficiency (Langlais and Zhang, 1993; Hazell et al., 1993). These studies are particularly relevant because thiamine deficiency is a critical factor in the etiology of Wernicke's encephalopathy and Korsakoff's amnesia, two disorders that are frequently associated with long-term ethanol intoxication in humans. However, dietary factors are unlikely to contribute to the changes in glutamate observed in our study for several reasons. Firstly, withholding of the normal diet for 6 days in control animals had no effect on extracellular glutamate. Secondly, abnormal glutamate levels were confined to the thalamus among structures examined (Langlais and Zhang, 1993; Hazell et al., 1993). Finally, the reduction of extracellular glutamate by ethanol or dizocilpine was reversible. Thus, the increase in extraneuronal glutamate more likely reflects overactivity of the glutamatergic system rather than neurotoxic processes. Nonetheless, it is possible that recurrent transient increases in extracellular glutamate concentrations, associated with repeated withdrawal episodes, could eventually result in glutamate-induced neurotoxic effects.

In conclusion, these results show that the increased extracellular glutamate represents a neurochemical correlate of ethanol withdrawal. They may also provide a biochemical rationale for the use of NMDA receptor antagonist drugs and particularly ethanol itself in the treatment of the acute phase of this excitatory syndrome.

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