UPREGULATION OF GLUTAMATE RECEPTOR SUBTYPES DURING ALCOHOL WITHDRAWAL IN RATS

STEVEN ROSENZWEIG HAUGBØL1, BJARKE EBERT2 and JAKOB ULRICHSEN1*1

1Neuropsychiatric Research Group, Department of Psychiatry 6234, University Hospital Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark and 2Department of Neurobiology, Biological Research, Lundbeck A/S, Ottiliavej 9, DK 2500 Valby, Denmark

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Abstract — Aims: To investigate glutamate receptor subtypes during alcohol withdrawal. Methods: Rats were exposed to severe alcohol intoxication for 84 h and then decapitated at 0, 12 and 36 h after the last alcohol dose (n = 7 per group). Alcohol was administered five times a day by intragastric intubation. The densities of N-methyl-d-aspartate (NMDA) and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors were studied in membranes from the forebrain by using the specific ligands [3H]MK-801 and [3H]AMPA, respectively. Results: Although no change in the maximal density (Bmax) of [3H]MK-801 binding sites was observed at the time of withdrawal, [3H]MK-801 binding was increased by 49% 12 h into the withdrawal reaction compared with the control group. At 36 h post alcohol the Bmax of the [3H]MK-801 binding was still increased by 24% compared with the control group; however, this difference was not statistically significant. When investigated at the time of withdrawal from chronic alcohol intoxication, no significant alterations in the Bmax of the [3H]AMPA binding was detected, but 12 h into the withdrawal reaction the [3H]AMPA binding was markedly increased by 94%. At 36 h post alcohol the [3H]AMPA binding had returned to control levels. No significant alterations in the dissociation constant (Kd) of either [3H]MK-801 or [3H]AMPA binding was observed at any time point. Conclusions: NMDA and AMPA receptors are involved in the cerebral hyperactivity of alcohol withdrawal.

INTRODUCTION

The amino acid glutamate is quantitatively the most important excitatory neurotransmitter in the central nervous system (CNS). It exerts its effects through specific receptors, which can be categorized into two groups, i.e. the metabotropic and ionotropic receptors. The metabotropic receptors are coupled to intracellular G proteins and when activated, they are involved in the production of secondary messengers in the activated neuron. The ionotropic receptors are ion channels, which, upon activation, allow a transmembranous passage of cations such as sodium, potassium and calcium, which results in a lowering of the membrane potential. The ionotropic glutamate receptors are further divided into the subtypes 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-d-aspartate (NMDA) and kainic acid according to their selective agonists (Watkins et al., 1990; Nakanishi, 1992; Sommer and Seeburg, 1992). For more than a decade several studies have indicated that glutamate may play an important role in the effects of alcohol in the brain including hyperactivity, which is seen during withdrawal from longterm intoxication. When administered acutely, alcohol in pharmacologically relevant concentrations causes a suppression of the NMDA receptor function in vitro. This has been demonstrated in electrophysiology studies in which NMDA-activated currents were inhibited by alcohol (Lovinger et al., 1989, 1990; White et al., 1990) and in biochemical experiments where alcohol resulted in inhibition of NMDA-induced Ca2+ uptake (Dildy and Leslie, 1989; Hoffman et al., 1989). On the other hand, when exposed chronically to alcohol the NMDA receptor function seems to be increased, perhaps as a result of adaptation to the inhibiting effects of acute alcohol administration. Thus, chronic alcohol administration resulted in increased ligand binding to the NMDA receptor (Grant et al., 1990; Gulya et al., 1991; Sanna et al., 1993; Hu and Ticku, 1995) and enhanced NMDA-stimulated increase in intracellular Ca2+ concentration (Iorio et al., 1993; Blevins et al., 1995; Hu and Ticku, 1995; Smoother et al., 1997). In addition, administration of NMDA to animals withdrawing from chronic alcohol exposure results in an increase in seizure activity (Grant et al., 1990; Sanna et al., 1993), mortality (Davidson et al., 1993; Sanna et al., 1993) and morphological damage (Davidson et al., 1995) compared with control animals, whereas the NMDA antagonist dizocilpine (MK-801) has been shown to inhibit alcohol withdrawal seizures in mice (Grant et al., 1990) and rats (Morrisett et al., 1990). Furthermore, mice bred to express a severe reaction to alcohol withdrawal have an increased density of NMDA receptor binding sites in hippocampus compared with mice bred to express a mild alcohol withdrawal reaction (Valverius et al., 1990).

The involvement of the AMPA and kainic acid receptors in the cerebral effects of alcohol has not been studied to the same extent as the NMDA receptor and the results are less consistent. Martin et al. (1995) demonstrated that alcohol inhibited AMPA and kainic acid induced depolarization in hippocampus. A similar finding was shown by Lovinger et al. (1989) but in this study the NMDA receptors were inhibited to a much higher degree by alcohol than the AMPA and kainic acid receptors. An increased sensitivity to NMDA and AMPA on the firing rate of the neurons was detected in alcohol withdrawing rats in the locus coeruleus, whereas the sensitivity to kainic acid was unchanged as compared with control animals (Engberg and Hajas, 1992). Binding studies in rats have shown that the densities of AMPA and kainic acid receptors in hippocampus were unaffected by a single episode of chronic alcohol intoxication (Rudolph et al., 1997), whereas kindling by alcohol withdrawal decreased the regional AMPA.
binding without affecting the regional densities of the kainic acid receptor (Ulrichsen et al., 1996).

Although the majority of the studies that have been conducted recently are in agreement with the theory that chronic alcohol administration results in an increased NMDA receptor function, there seems to be disagreement about the time course of the receptor alterations that, in part, may be due to interspecies variations. Thus, in mice, the density of the NMDA receptor seems to be upregulated at the time at which alcohol administration is terminated (Grant et al., 1990; Gulya et al., 1991), i.e., the changes take place during the alcohol intoxication period, whereas in rats it was shown that the NMDA receptor binding was unchanged at the end of the alcohol intoxication period but increased markedly during the subsequent withdrawal reaction (Sanna et al., 1993). In order to shed more light on these inconsistent results and to study the AMPA receptor in alcohol dependence further, the present binding experiment was performed to study the time course of the NMDA and AMPA receptors during the alcohol withdrawal reaction in rats.

MATERIALS AND METHODS

Animals and intoxication procedure

Eighty male Wistar rats (Møllegaard, Køge, Denmark) were housed in a room with a 12 h light/dark cycle (lights on at 07:00 h) and had free access to food pellets and water. The body weight was initially 180–200 g. The animals were exposed to severe alcohol intoxication for 84 h. Alcohol was administered five times a day between 08:00 h and 24:00 h by the intragastric intubation method (Majchrowicz, 1975). Before each feeding session the degree of alcohol intoxication was assessed by using the following rating scale.

(0) Neutrality: no signs of intoxication.
(1) Sedation: reduced muscle tone, dulled appearance and slow locomotor activity but no impairment of gait or coordination.
(2) Ataxia 1: slight gait impairment and slight motor incoordination but able to elevate abdomen and pelvis.
(3) Ataxia 2: clearly impaired staggering gait and impaired motor coordination, some elevation of abdomen and pelvis.
(4) Ataxia 3: slowed righting reflex, heavily impaired motor coordination, no elevation of abdomen and pelvis.
(5) Loss of righting reflex (LRR): unable to right itself when placed on its back, other reflexes still present.
(6) Coma: no signs of movement; no response to pain stimuli; no blinking reflex; spontaneous breathing.

The alcohol dose was adjusted individually according to the degree of intoxication. Neutral rats received 5–7 g/kg whereas animals with LRR received 0–1 g/kg. The aim was to reach an intoxication level of 3–5 g (ataxia 2–LRR) within 8 h and to keep this level for the rest of the intoxication period. The alcohol solution consisted of: 200 g/l of ethanol, 300 g/l of sucrose and 4 ml/l of multivitamin mixture in Ringer’s solution, the sucrose being added in order to prevent hypoglycaemia and ketosis (Hemmingsen and Chapman, 1980). Control animals received an isocaloric amount of sucrose instead of alcohol and the same amount of water and food as the alcohol treated rats. The blood concentration of alcohol was not measured in the present study, but previous studies have shown that it is 3–5 g/l during most of the intoxication period (Majchrowicz, 1975; Ulrichsen et al., 1995, 1998).

Nine animals died due to alcohol intoxication. After the last alcohol dose, 21 of the 71 surviving alcohol treated animals were randomly selected for glutamate receptor binding and the remaining animals were used in other experiments. The 21 animals were randomly allocated to three groups (n = 7 per group), which were decapitated at 0, 12 or 36 h after the last alcohol dose, respectively. After decapitation, which was performed under light halothane anaesthesia, the brains were rapidly removed and frozen in isopentane cooled by a bath of acetone and dry ice (−80°C). The whole brains were stored at −80°C. The control animals (n = 7) were decapitated 12 h after the last alcohol dose was administered to the experimental animals.

Before the animals were decapitated, the severity of the withdrawal reaction was assessed openly by scoring the three individual items: intentional tremor, rigidity and hyperactivity/irritability on a 4 level scale (0–3). The sums of these scores (0–9) were used as a quantitative measurement of the severity of the withdrawal reaction (Ulrichsen et al., 1986). The level of alcohol intoxication was measured by using the rating scale mentioned above (0–6).

All animals were weighed immediately before the first and last alcohol dose.

Membrane preparation

The whole brains were thawed and the cerebellum and midbrain removed. The remaining cerebrum was divided longitudinally and each half was used for either [3H]MK-801 binding or [3H]AMPA binding. Tissue preparation was performed as described by Ransom and Stec (1988). Briefly, the tissue was homogenized (500 r.p.m.) in 10 volumes (w/v) of ice-cold 0.32 M sucrose by eight strokes of a glass-teflon homogenizer. The homogenate was centrifuged at 1000 g for 10 min and the supernatant was then centrifuged at 20 000 g for 20 min at 4°C. The resulting pellet was then resuspended in 20 volumes of ice-cold distilled water and homogenized (100 r.p.m.) with a glass-teflon homogenizer. The homogenate was centrifuged at 8000 g for 20 min at 4°C. The supernatant and buffy coat was decanted and recentrifuged at 48 000 g for 20 min at 4°C. The pellet was resuspended in 20 volumes of ice-cold distilled water and centrifuged at 48 000 g for 20 min at 4°C. The last step was repeated once and the pellet was frozen at −20°C for at least 18 h.

On the day of the assay, the membrane pellet was thawed at room temperature for 45 min, suspended in 75 volumes of 5 mM Tris–HCl buffer (pH 7.4 at 25°C) using a thurax homogenizer (2 × 10 s) and centrifuged for 20 min (48 000 g) at 4°C. This step was repeated three times. The membranes were resuspended in the assay buffer prior to the final centrifugation step.

[3H]MK-801 binding

[3H]MK-801 binding was performed with modifications as described previously (Ransom and Stec, 1988). The assay was carried out in a volume of 1 ml. This volume was made up of: 750 µl of membrane suspension; 100 µl of [3H]ligand; 100 µl...
of test substance and 50 µl of buffer or modulator. Determinations were carried out in triplicate.

Well washed membranes were resuspended in 5 mM Tris–HCl buffer (pH 7.4) corresponding to ~12 mg of original tissue per well. Binding experiments were carried out at 25°C and under maximum stimulation, i.e. a final concentration of 30 µM (S)-glutamate and 1 µM glycine. Construction of a saturation curve was carried out using a combined increase of radioactive ligand and isotope dilution (Ebert et al., 1991). Radioligand concentrations between 0.1 and 3 nM were obtained by increasing concentrations of [3H]MK-801 (specific activity 22.5 Ci/mmol), whereas concentrations >3 nM were obtained by isotope dilution of 3 nM [3H]MK-801 with non-radioactive MK-801 (total ligand concentration ranged from 0.1 to 12 nM). Non-specific bound [3H]MK-801 was determined using 100 µM 1-(2-thienyl)cyclohexyl piperidine (TCP).

After incubation for 4 h, binding was determined by filtration through Whatman GF/B filters (presoaked for at least 2 h in 0.1% polyethylenimine solution) using a Brandell M-48R cell harvester and washed three times using 2 ml of ice-cold buffer.

For each total MK-801 concentration (i.e. radioactive and non-radioactive) specific bound ligand was calculated as described previously (Honore and Nielsen, 1985). The assay was carried out in a volume of 250 µl. This volume was made up of: 200 µl of membrane suspension; 25 µl of [3H]-ligand and 25 µl of test substance. Determinations were carried out in triplicate.

Well washed membranes were resuspended in 30 mM Tris–HCl (pH 7.1) containing 2.5 mM CaCl₂ and 100 mM potassium thiocyanate (KSCN) corresponding to ~9 mg original tissue per well. The binding experiments were carried out at 0–4°C. Construction of a saturation curve was carried out using a combined increase of radioactive ligand and isotope dilution (Ebert et al., 1991). Radioligand concentrations between 1 and 9 nM were obtained by increasing concentrations of [3H]AMPA (specific activity 52 Ci/mmol), whereas concentrations >9 nM were obtained by isotope dilution of 9 nM [3H]AMPA with non-radioactive AMPA (total ligand concentration ranged from 1 to 109 nM). Non-specific binding was determined using 1 mM (S)-glutamate. After incubation for 4 h, bound ligand was separated from free using a Brandell M-48R cell harvester and washed three times using 2 ml of ice-cold buffer.

For each total AMPA concentration (i.e. radioactive and non-radioactive) specific bound ligand was calculated as described previously (Ebert, 1991). Radioligand concentrations between 1 and 9 nM were obtained by increasing concentrations of [3H]AMPA (specific activity 52 Ci/mmol), whereas concentrations >9 nM were obtained by isotope dilution of 9 nM [3H]AMPA with non-radioactive AMPA (total ligand concentration ranged from 1 to 109 nM). Non-specific binding was determined using 1 mM (S)-glutamate. After incubation for 4 h, bound ligand was separated from free using a Brandell M-48R cell harvester and washed three times using 2 ml of ice-cold buffer.

For each total AMPA concentration (i.e. radioactive and non-radioactive) specific bound ligand was calculated as described previously (Ebert, 1991). Based on the resulting saturation curves Bₘₐₓ and Kᵣ were determined for each animal using the non-linear curvefitting program Grafit 5.0 (Erithacus Software, Staines, UK).

### [3H]AMPA binding

[3H]AMPA binding was performed with modifications as described previously (Honore and Nielsen, 1985). The assay was carried out in a volume of 250 µl. This volume was made up of: 200 µl of membrane suspension; 25 µl of [3H]-ligand and 25 µl of test substance. Determinations were carried out in triplicate.

Well washed membranes were resuspended in 30 mM Tris–HCl buffer (pH 7.4) containing 2.5 mM CaCl₂ and 100 mM potassium thiocyanate (KSCN) corresponding to ~12 mg of original tissue per well. The binding experiments were carried out at 0–4°C. Construction of a saturation curve was carried out using a combined increase of radioactive ligand and isotope dilution (Ebert et al., 1991). Radioligand concentrations between 1 and 9 nM were obtained by increasing concentrations of [3H]AMPA (specific activity 52 Ci/mmol), whereas concentrations >9 nM were obtained by isotope dilution of 9 nM [3H]AMPA with non-radioactive AMPA (total ligand concentration ranged from 1 to 109 nM). Non-specific binding was determined using 1 mM (S)-glutamate. After incubation for 4 h, bound ligand was separated from free using a Brandell M-48R cell harvester and washed three times using 2 ml of ice-cold buffer.

For each total AMPA concentration (i.e. radioactive and non-radioactive) specific bound ligand was calculated as described previously (Ebert, 1991). Radioligand concentrations between 1 and 9 nM were obtained by increasing concentrations of [3H]AMPA (specific activity 52 Ci/mmol), whereas concentrations >9 nM were obtained by isotope dilution of 9 nM [3H]AMPA with non-radioactive AMPA (total ligand concentration ranged from 1 to 109 nM). Non-specific binding was determined using 1 mM (S)-glutamate. After incubation for 4 h, bound ligand was separated from free using a Brandell M-48R cell harvester and washed three times using 2 ml of ice-cold buffer.

For each total AMPA concentration (i.e. radioactive and non-radioactive) specific bound ligand was calculated as described previously (Ebert, 1991). Based on the resulting saturation curves Bₘₐₓ and Kᵣ were determined for each animal using the non-linear curvefitting program Grafit 5.0 (Erithacus Software, Staines, UK).

### Ligands

[3H]MK-801 and TCP was purchased from Sigma, Copenhagen, Denmark and [3H]AMPA was kindly delivered by Professor Povl Krosggaard-Larsen, from Danish University of Pharmaceutical Sciences.

### Statistical analysis

Binding was first investigated by one-way analysis of variance (ANOVA). If a significant group effect was found, the experimental groups were subsequently compared with the control group by two sided t-tests. Data are presented as mean (SEM). The level of significance was set everywhere to 5%.

### Ethics

The study was approved by the Danish Animal Experiment Inspectorate, Ministry of Justice.

### RESULTS

The alcohol dose, mean intoxication score and the intoxication and withdrawal score at the time of decapitation is shown in Table 1. All experimental animals were severely intoxicated during the alcohol intoxication phase. A weight loss of 13% (0.4) was observed in the control animals, whereas the alcohol-treated animals decapitated at 0, 12 and 36 h post alcohol had a weight loss of 15% (0.5%), 14% (0.7%) and 13% (0.5%), respectively.

Kᵣ and Bₘₐₓ for cerebral [3H]MK-801 binding to NMDA receptors in alcohol-dependent rats and isocalorical fed controls are shown in Table 2. No significant group effects were found for the dissociation constant Kᵣ, whereas ANOVA revealed a significant group effect for the density of NMDA receptors. [3H]MK-801 binding was not affected by chronic alcohol intoxication per se, but during the withdrawal period in rats decapitated during alcohol intoxication or during the alcohol withdrawal reaction at 12 or 36 h post alcohol.

**Table 1.** Alcohol intoxication parameters for the 84 h intoxication period in rats decapitated during alcohol intoxication or during the alcohol withdrawal reaction at 12 or 36 h post alcohol

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intoxication (n = 7)</th>
<th>Withdrawal (12 h) (n = 7)</th>
<th>Withdrawal (36 h) (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily ethanol dose (g/kg/24 h)</td>
<td>12.6 (0.3)</td>
<td>11.8 ± 0.2</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>Mean intoxication score</td>
<td>2.9 (0.05)</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.05</td>
</tr>
<tr>
<td>Intoxication score at decapitation (0–6)</td>
<td>2.7 (0.1)</td>
<td>1.2 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Withdrawal score at decapitation (0–9)</td>
<td>0.0 (0.0)</td>
<td>2.8 ± 0.4</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM.

**Table 2.** [3H]MK801 binding in cerebrum of rats exposed to 84 h severe alcohol intoxication; the rats were decapitated during alcohol intoxication or during the alcohol withdrawal reaction at 12 or 36 h post alcohol

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Kᵣ (nM)</th>
<th>Bₘₐₓ (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.99 ± 0.2</td>
<td>6.17 ± 0.5</td>
</tr>
<tr>
<td>Intoxication</td>
<td>6</td>
<td>1.19 ± 0.06</td>
<td>5.97 ± 0.3</td>
</tr>
<tr>
<td>Withdrawal (12 h)</td>
<td>5</td>
<td>1.23 ± 0.03</td>
<td>9.21 ± 0.6*</td>
</tr>
<tr>
<td>Withdrawal (36 h)</td>
<td>6</td>
<td>1.23 ± 0.06</td>
<td>7.68 ± 0.7</td>
</tr>
</tbody>
</table>

ANOVA: Non significant

Data represent mean ± SEM. A statistical significant difference from control value is indicated by *P < 0.05 (t-test).
reaction, binding of $[^3]$HMK-801 was upregulated. Thus, 12 h into withdrawal a significant increase (of 49%) in the density of NMDA binding sites was detected compared with the control level. In the animals that were decapitated 36 h into the withdrawal reaction, the density of NMDA binding sites was still increased compared with the control animals (by 24%), but the difference did not reach statistical significance ($P = 0.1$).

The $K_D$ and $B_{\text{max}}$ for cerebral $[^3]$HAMP A binding in alcohol-dependent rats and isocaloric fed controls are summarized in Table 3. $K_D$ did not differ across groups. A significant group effect was observed for the $B_{\text{max}}$ of AMPA binding sites. The density of AMPA binding sites was not affected by chronic alcohol intoxication per se, but 12 h into withdrawal this variable was increased significantly (by 94%) compared with the control animals. Later during the withdrawal reaction, i.e., 36 h post alcohol, the density of AMPA binding sites had returned to control levels.

**DISCUSSION**

The upregulation of the NMDA receptors during the withdrawal reaction found in the present study is consistent with the theory that the cerebral hyperactivity, which follows long-term alcohol intoxication, in part is due to an excessive stimulation of the NMDA receptors (Glue and Nutt, 1990). Although we detected no alterations when the animals were still intoxicated, the density of the NMDA binding sites was increased by 49% 12 h into the withdrawal reaction. During the next 24 h the enhancement of binding sites was reduced to a 24% increase compared with control levels; a difference which did not reach statistical significance. This time course is in agreement with the study by Sanna et al. (1993) in which an autoradiography study an upregulation of MK-801 binding was demonstrated in hippocampus, cortex, striatum and thalamus in mice at the time of withdrawal (Gulya et al., 1991). In this study, the time course of changes in MK-801 binding in hippocampal membranes showed that the increased NMDA binding persisted 10 h into the withdrawal reaction whereas the NMDA receptors had returned to control levels 20 h after the last alcohol dose (Gulya et al., 1991). In these experiments, alcohol was administered as liquid diet to the mice, a technique which does not provide quite the same blood alcohol concentration as the intragastric intubation method of Majchrowicz in which maximal tolerable doses of alcohol is administered (Majchrowicz and Hunt, 1976). Although the discrepancies in the time course of NMDA receptor binding between the current study and Sanna et al. (1993) on the one hand and Grant et al. (1990), Valverius et al. (1990) and Gulya et al. (1991) on the other hand could theoretically be due to differences in alcohol administration, we find it more likely that they are brought about by interspecies variation. It is therefore important to investigate species other than rodents in order to understand the role of the NMDA receptor in alcohol-dependent humans.

An earlier autoradiography experiment from our laboratory does not agree with the current results, as we failed to detect regional changes in $[^3]$HMK-801 binding in rats exposed to severe alcohol intoxication for 2 days and investigated 10–12 h after the last alcohol dose (Ulrichsen et al., 1996). As the withdrawal reaction is much more severe after 4 days rather than 2 days of severe alcohol intoxication (Ulrichsen et al., 1998), the NMDA receptor may not be changed if the withdrawal reaction is relatively mild. The seizure component of the withdrawal reaction may be particularly related to an increased density of NMDA binding sites. Thus, seizures are not observed in rats exposed to severe alcohol intoxication for 2 days while 25–35% show spontaneous withdrawal seizures after 4 days of alcohol intoxication (Ulrichsen et al., 1996, 1998).

It has been postulated that physical dependence on alcohol is developed as a cerebral response to the depressant effects of alcohol in order that the brain may restore normal function despite the presence of alcohol (i.e., tolerance) and that when the suppression of the brain activity is terminated by
withdrawal of alcohol, these cerebral alterations give rise to a rebound hyperactivity in the brain (Kalant et al., 1971). This theory is elegant and easily understood but it may not be applicable to the glutamatergic neurotransmission, as the current results suggest that upregulation of glutamate receptor subtypes specifically occurs during the withdrawal phase rather than the preceding episode of intoxication. It has been shown using microdialysis that intracerebral administration of NMDA in vivo results in a marked increase in extracellular glutamate concentration. Thus, Rossetti et al. (1999) showed that in saline treated control animals the glutamate concentration in striatum was increased to 268% of baseline values after NMDA administration. In alcohol-dependent animals investigated at the time of withdrawal no effect was observed, while 12 h into the withdrawal reaction, NMDA administration resulted in a 6-fold (598%) increase of the baseline glutamate levels. The increase in glutamate output was blocked by the non-competitive NMDA receptor antagonist MK-801. Assuming that the presence of alcohol at the time of withdrawal inhibited the NMDA receptors (Dildy and Leslie, 1989; Hoffman et al., 1989; Lovinget et al., 1989, 1990; White et al., 1990), the results of Rossetti et al. (1999) are in agreement with the present binding results and similarly to our findings, they also suggest that the supersensitivity of the NMDA receptor is developed during the withdrawal phase rather than during the period of intoxication. Alcohol affects a variety of neurotransmitters of which, gamma-aminobutyric acid (GABA) along with glutamate, are probably the most widely studied. In accordance with Morrow et al. (1988), Sanna et al. (1993) detected a reduced ability of GABA and flunitrazepam to stimulate 36Cl− uptake in cortical vesicles prepared from neurons of alcohol-dependent rats when still intoxicated. However, when the animals showed clinical signs of withdrawal, the GABA- and flunitrazepam-induced 36Cl− uptake had returned to control levels (Sanna et al., 1993). These results indicate that the GABA<sub>A</sub> receptor is involved in alcohol tolerance but not in the cerebral hyperactivity of the alcohol withdrawal reaction. Upon withdrawal of alcohol, the abnormal function of GABA (and other neurotransmitter systems involved in tolerance) returns to habitual conditions (Sanna et al., 1993), but it is likely that this process of restoring the impaired GABAergic inhibition results in secondary CNS alterations, for instance, in the activity of the glutamatergic neurotransmission. Hence, the upregulation of the density of NMDA binding sites in the present study may represent an attempt by the brain to counteract alterations, which occur in GABAergic (and other neurotransmitter) systems involved in alcohol tolerance, as the overall CNS inhibition by GABA is increased after alcohol is withdrawn. Therefore, the cerebral mechanisms of the alcohol withdrawal reaction may not be directly related to tolerance as suggested by Kalant et al. (1971), but rather develop secondarily to the process of tolerance reduction upon termination of alcohol intake.

Experiments using genetic animal models provide further evidence for an involvement of the NMDA receptor in the alcohol withdrawal reaction but not in the mechanisms of alcohol tolerance. In mice that were selectively bred to be tolerant to alcohol no difference in central MK-801 binding was detected compared with non-tolerant animals (Näkki et al., 1995) whereas mice bred to be sensitive to alcohol withdrawal seizures exhibited an upregulation of MK-801 binding sites in hippocampus compared with the seizure resistant line (Valverius et al., 1990).

Cloning studies have shown that the NMDA receptors are composed of multiple subunits designated NR1, NR2a, NR2b, NR2c and NR2d (or NMDAR1, NMDAR2a,b,c,d). The NR1 subunit is required to generate functional NMDA receptors, whereas co-expression with various NR2 subunits results in the assembly of ion channels that resemble native NMDA receptors (reviewed by Davis and Wu, 2001). Recent studies have shown that chronic alcohol intoxication affects the NMDA receptor subunits and the mRNA encoding for these proteins. An increase in NR1 receptor subunit immuno-reactivity was found in membranes from whole brain (Chen et al., 1997) and hippocampus (Trevisan et al., 1994) of rats fed a liquid ethanol diet for 15 days and 12 weeks, respectively. Upregulation of NR1, NR2a and NR2b receptor subunits was detected in cerebral cortex and hippocampus (Kaluri et al., 1998) in rats intoxicated by the intragastric intubation method for 6 days. In a previous study from the same laboratory, an increase in the levels of the mRNA encoding for the NR2a and NR2b subunits was demonstrated, whereas the expression of NR1 mRNA was unchanged compared with the control animals (Follesa and Ticku, 1995). NR1 and NR2a proteins were upregulated in alcohol-dependent mice whereas no alterations were detected in the mRNA levels for these subunits (Snell et al., 1996). These results suggest that upregulation of NMDA receptors in alcohol-dependent rodents is brought about by an increase in both NR1 and NR2 protein subunits and that the mechanisms behind these changes may be different. In the former case, a decrease in receptor protein degradation may be responsible for the alterations whereas an increased mRNA formation leading to an elevated protein synthesis may result in increased expression of NR2a and NR2b receptor subunits. These speculations are tentative and more research into the regulation of the NMDA receptor subunits is needed to elucidate the mechanisms involved in the upregulation of NMDA receptors in alcohol dependence.

As was the case with the NMDA receptor, the AMPA receptor was also upregulated during the alcohol withdrawal reaction, and to an even larger extent, as the density of the AMPA binding was increased by 94% compared with the control group. Therefore this receptor may also be involved in the hyperactivity of alcohol withdrawal. The AMPA receptors conduct mainly Na⁺ currents and mediate fast excitatory synaptic transmission, whereas the NMDA receptors display high Ca²⁺ permeability and voltage-dependent Mg²⁺ block. Since Mg²⁺ blocks the ion channel the NMDA receptor is relatively insensitive to glutamatergic and aspartergic stimulation at resting membrane potential, but as the membrane potential is decreased, for instance, by activation of AMPA receptors, Mg²⁺ causes less blockade, allowing for an increase in Ca²⁺ influx and further depolarization upon agonist activation (Nakanishi, 1992; Sommer and Seeburg, 1992). Therefore, the upregulation of the NMDA and AMPA receptors during the withdrawal reaction may act synergistically to increase the excitatory neurotransmission. To our knowledge, this is the first study in which upregulation of AMPA binding sites in alcohol dependence has been demonstrated. In one
ligand binding experiment in rats in which several alcohol paradigms were employed, no changes in AMPA binding were observed (Rudolph et al., 1997), but as all binding experiments were conducted on animals that were still intoxicated, the negative results are in agreement with our current findings. No change in AMPA binding densities was observed (Freund and Anderson, 1996) in human alcoholics who had been sober for at least 2 weeks. In contrast to the present results, we did not find significant changes in regional [3H]AMPA binding in rats exposed to 2 days of severe alcohol intoxication investigated 10–12 h into the withdrawal reaction compared with sucrose treated controls (Ulrichsen et al., 1996). However, in the same study, alcohol withdrawal kindled rats exposed to multiple episodes of alcohol intoxication and withdrawal showed a significant downregulation of [3H]AMPA binding in several regions, perhaps as an adaptive response to kindling induced cerebral hyperactivity. In consensus with the current results it was shown in an electrophysiology study that the sensitivity to AMPA was increased in locus coeruleus of alcohol withdrawing rats (Engberg and Hajos, 1992). Obviously the mechanisms by which these receptors are regulated during alcohol withdrawal are different.

In conclusion, the present study showed that the densities of NMDA and AMPA binding sites were upregulated during the withdrawal reaction, whereas no changes were observed at the time of withdrawal. These alterations may play an important role in the cerebral hyperactivity of the withdrawal reaction.

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