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Short communication

Long-term voluntary ethanol drinking increases expression of NMDA receptor 2B subunits in rat frontal cortex

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Abstract

Forced ethanol drinking for several days and application of ethanol to cell cultures changes expression levels of various NMDA receptor subunits in rodents. We investigated the influence of long-term voluntary ethanol consumption of moderate ethanol doses on polypeptide levels of the NMDA receptor subunit 2B (NR2B) in various forebrain regions of rats and found a mild increase selectively in the frontal cortex. This result is in accordance with and extends findings from studies using high doses of ethanol for a short period and suggests that the NR2B might be a potential target for an effective treatment of alcoholic patients.

Keywords: Alcohol; Self-administration; NR2B subunit; Protein; Up-regulation

1. Introduction

There is substantial evidence that the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor represents a particularly important site of action for ethanol in the central nervous system. Ethanol acutely inhibits NMDA-induced responses, whereas chronic ethanol treatment exerts an enhancing effect on the NMDA receptor function (for review see Kumari and Ticku, 2000). Multiple mechanisms may contribute to chronic ethanol-induced alterations in NMDA receptor function. One of those mechanisms might be the differential regulation of the expression of NMDA receptor subunits. Thus, in rodents NMDA receptor subunit expression was differentially altered in cell cultures of cortical neurons and tissue of various brain regions after chronic ethanol treatment, both on mRNA and protein levels (Chandler et al., 1999; Follesa and Ticku, 1995, 1996b; Hu et al., 1996; Kalluri et al., 1998; Narita et al., 2000; Snell et al., 1996). In these studies, however, "chronic" ethanol treatment is either defined as the application of rather high doses of ethanol to cell culture or as forced ethanol intake of rats or mice, for 1 or 2 weeks. In humans, this procedure would, to some extent, correspond to severe ethanol intoxication over a period of several days, whereas long-term voluntary ethanol intake of lower doses might reflect a more prevalent human alcohol drinking behaviour, possibly preceding harmful alcohol consumption.

Therefore, we investigated the influence of long-term voluntary ethanol consumption on the expression of NMDA receptor 2B and (NR2B) subunits in several forebrain regions of rats. The NR2B subunit is primarily expressed in the forebrain (Wang et al., 1995; Wenzel et al., 1995). Several lines of evidence particularly imply the NR2B subunit in the development of ethanol dependence: NR2B subunits are involved in the mediation of the ethanol sensitivity of native and recombinant NMDA receptors (Engblom et al., 1997; Lovinger, 1995), and in vivo inhibitory electrophysiological effects of ethanol highly correlate with those of ifenprodil, a selective NR2B antagonist (Yang et al., 1996), which suppresses ethanol withdrawal after 5 days forced ethanol intake in mice (Narita et al., 2000). Furthermore, persistent stress-induced increase of alcohol drinking in corticotropin-releasing hormone receptor 1 (CRH1)-deficient mice is associated with a selective up-regulation of the NR2B subunit, without changes in any other ionotropic glutamate receptor subtype (Sillaber et al., 2002).

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2. Materials and methods

2.1. Animals

Male Wistar rats (Max Planck Institute of Biochemistry, Martinsried, Germany) weighing about 200 g at the beginning of the experiments were housed individually in standard type II Macrolon™ cages with food and water ad libitum. Artificial light was provided daily from 7:00 A.M. until 7:00 P.M.

The experiments were approved by the Committee on Animal Care and Use of the local governmental body.

2.2. Procedure

At the age of 6 weeks, rats were randomly assigned to two groups: one group continued to have free access to tap water (water group, $n=7$), the other group was given free access to 5%, 10% and 20% (v/v) ethanol solutions in addition to tap water in the home cages (ethanol group, $n=5$). For a period of 19 months rats of the ethanol group consumed an average of 2 ± 0.5 g ethanol per kg body weight and day; the ethanol preference (defined as the percentage share of the sum of consumption from the three ethanol solutions in total fluid consumption) was between 30% and 40%. After 19 months, immediately before decapitation, all rats were exposed to 15 min of restraint stress, as the animals served as controls in another experiment (regarding the effect of repeated stress experience on voluntary ethanol intake in long-term ethanol experienced rats). For decapitation rats were slightly anaesthetised with isoflurane (Curamed, Karlsruhe, Germany). Trunk blood was collected to determine blood ethanol levels. The brains were rapidly removed, shock-frozen in *n*-methylbutane on dry ice and stored at -80 °C.

2.3. Immunoblotting

Brains were dissected into frontal cortex, nucleus accumbens, caudatoputamen and hippocampus. For this purpose the frozen brains were sectioned in a cryocut (Microm HM 500 OM, Walldorf, Germany) into slices of various thickness (according to the stereotaxic atlas of Paxinos and Watson (1986) and relative to bregma: frontal cortex 5.2 to 2.7 mm, nucleus accumbens 2.7 to 0.7 mm, caudatoputamen 0.7 to -1.3 mm, hippocampus -2.3 to -5.3 mm). From those slices the respective brain regions were dissected bilaterally using a scalpel or punch (nucleus accumbens), respectively.

Each of the brain regions was homogenised separately for each rat in ice cold membrane buffer (50 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 1 mM EGTA, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine) by repeated pipetting through small cannulae (23 G). The homogenate was centrifuged twice at $33{,}000 \times g$ for 15 min at 4 °C and the precipitate was used as membrane protein sample. Protein concentration was determined with the Bio-Rad DC protein assay kit (Bio-Rad, Munich, Germany). Equal quantities of protein (25 μg) were separated by 9% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes (0.45 μm, Schleicher and Schüll, Dassel, Germany), using a Mini Transfer Cell (Bio-Rad). The nitrocellulose membranes were then blocked with 5% bovine serum albumin and incubated overnight with the primary antibody (rabbit anti-NMDAR2B; Chemicon, Temecula, CA, USA; dilution 1:5,000) at room temperature. Incubation with the secondary antibody (donkey peroxidase-linked anti-rabbit immunoglobulin G; Amersham Buchler, Braunschweig, Germany; dilution 1:10,000) was performed for 2 h at room temperature. All antibody incubations, washes and dilutions were carried out in Tris-buffered saline containing 0.1% Tween 20. Antibody detection was performed with the Amersham ECL Western blotting analysis system and the signal was exposed to Hyperfilm-ECL (Amersham Buchler). For control of protein amounts, samples (25 μg) were run on a 9% SDS-PAGE and the gels were stained with Coomassie Briliant Blue. Unless stated otherwise, all chemicals were obtained from Sigma (Deisenhofen, Germany).

Each gel comprised samples of the two experimental groups. Analysis was conducted by measuring the optical density of the bands (arbitrary units) using an Optimas image analysis system (Optimas/BioScan, Edmonds, USA).

2.4. Statistical analysis

For each animal and brain region at least three blots were prepared and averaged after analysis. The data were normalised to the amount of protein loaded in each lane in the Coomassie blue-stained gel. The value of each animal was calculated as percentage of the mean value of the

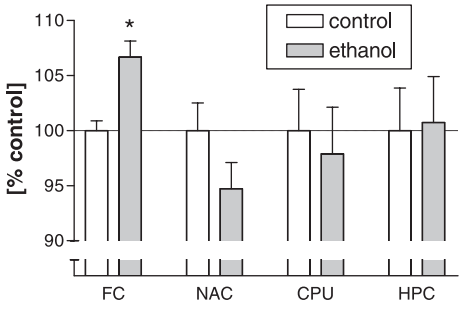

Fig. 1. Regional protein levels of NR2B subunit in water drinking control ($n=7$) and long-term ethanol experienced ($n=5$) rats. Bars represent means (± S.E.M.) of optical-density grey values normalised for the averaged control values of the respective brain regions (100%). FC = frontal cortex, NAC = nucleus accumbens, CPU = caudatoputamen, HPC = hippocampus. *$P<0.05$ vs. control group, Mann–Whitney *U* test.

water control group (taken as 100%). Comparison of data between the ethanol group and the water control group was made by Mann–Whitney *U* tests. A two-tailed probability of less than 5% ($P < 0.05$) was accepted as statistically significant.

3. Results

Chronic ethanol consumption resulted in a 7% increase of NR2B polypeptide in frontal cortex ($P < 0.05$, Mann–Whitney *U* test). There were no significant differences in NR2B polypeptide levels between long-term ethanol-experienced and water drinking control rats in the nucleus accumbens, caudatoputamen or hippocampus (Fig. 1).

4. Discussion

Long-term voluntary ethanol intake of approximately 2 g/kg/day ethanol resulted in a mild up-regulation of NR2B in frontal cortex but not in nucleus accumbens, caudatoputamen or hippocampus. Similar to our results an increase of NR2B subunits selectively in cerebral cortex, but not in hippocampus and hypothalamus, has been shown in rats after 14 days of forced ethanol consumption of about 10–12 g/kg/day (Devaud and Morrow, 1999). An elevated expression of NR2B subunits has also been observed in cultures of mouse and rat cortical neurons after 5 to 12 days of alcohol exposure (Chandler et al., 1999; Follesa and Ticku, 1996b; but see also Narita et al., 2000). However, the mechanism by which ethanol alters NMDA receptor expression in cultured neurons, where receptors are undergoing development, probably differs from the mechanism of the ethanol's effect on NMDA receptors in adult brain (Snell et al., 2001).

It is unlikely that our results are confounded by the restraint stress carried out immediately before decapitation, as the 15-min restraint time period would be too short to result in altered protein expression. Most likely, the up-regulation of NR2B subunits in the frontal cortex reflects a cellular adaptation to the prolonged inhibitory effect of ethanol on NMDA receptors. This notion is supported by two studies that found an increase in the levels of the NR2B mRNA (Follesa and Ticku, 1996a) and polypeptide (Follesa and Ticku, 1996b) after chronic (5 days) exposure of mouse cortical neurons to the NMDA receptor antagonists D(−)-2-amino-5-phosphonopentanoic acid (AP5) and (+)-3-2-(carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), respectively.

Recent research in humans using brain imaging, neuropsychological testing and other techniques has revealed that the frontal lobes are particularly vulnerable to the depressant effects of low to moderate doses of ethanol (for review see Lyvers, 2000). Furthermore, moderate increases of NMDA but not AMPA and kainate subtypes of glutamate receptors have also been observed in agonist and antagonist binding studies in the frontal cortex of post-mortem tissue of chronic alcoholics (Freund and Anderson, 1996). As the up-regulation of NR2B in the frontal cortex might be an important factor underlying the development of withdrawal symptoms and/or the high probability of relapse, the NR2B subunit might be a promising target for an effective treatment of alcoholic patients.

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