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European Journal of Pharmacology 401 (2000) 191–195

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Short communication

Implications of the NR2B subunit-containing NMDA receptor localized in mouse limbic forebrain in ethanol dependence

Minoru Narita [a], Miho Soma [a], Michiko Narita [b], Hirokazu Mizoguchi [b], Leon F. Tseng [b], Tsutomu Suzuki [a,*]

[a] *Department of Toxicology, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*
[b] *Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*

Accepted 20 June 2000

Abstract

The present study was designed to further investigate the direct involvement of the NR2B-containing NMDA receptor in ethanol dependence. Using the liquid diet method, mice were chronically treated with skimmed milk containing 5% ethanol for 5 days. After the discontinuation of ethanol, mice revealed tremor, handling-elicited convulsion and death. Treatment with a selective NR2B-containing NMDA receptor antagonist, ifenprodil, significantly suppressed the expression of ethanol withdrawal signs. The protein level of NR2B subunits in the limbic forebrain, but not the cerebral cortex, during chronic ethanol treatment was markedly increased with respect to the levels in control mice. The significant up-regulation of NR2B subunits lasted for at least 9 h after the discontinuation of ethanol and returned to the basal level by 48 h after the withdrawal. These findings suggest that the up-regulation of NR2B subunits during chronic ethanol exposure may be implicated in the initial development of physical dependence on ethanol.

Keywords: Ethanol withdrawal; Ifenprodil; NMDA receptor subunit; Liquid diet method

1. Introduction

The process of ethanol dependence and withdrawal syndrome is thought to result from adaptive changes in a number of neurotransmission systems. It has been reported that chronic ethanol treatment increases the number of NMDA receptors in the brain, probably as a result of adaptive response to the depressant effects of acute ethanol treatment (Grant et al., 1990; Gulya et al., 1991). Administration of MK-801 (dizocilpine), a subunit non-selective NMDA receptor antagonist, has been shown to attenuate ethanol withdrawal seizures in mice and rats (Grant et al., 1990; Morrisett et al., 1990). Recently, molecular cloning studies have revealed that the NMDA receptor consists of two families, the NR1 and NR2 (NR2A-2D) subunits. Several lines of evidence have suggested that ifenprodil shows a high affinity for recombinant heteromeric NR1/NR2B-containing NMDA receptors (Williams, 1993). We previously reported that administration of either dizocilpine or ifenprodil suppressed the diazepam withdrawal signs in rats (Tsuda et al., 1998b), and that protein levels of the NR2B subunit were up-regulated during diazepam withdrawal (Tsuda et al., 1998a). In the present study, to clarify the role of NR1/NR2B-containing NMDA receptors in the expression of ethanol withdrawal signs, we examined the effect of ifenprodil on the spontaneous withdrawal signs caused by abrupt withdrawal from ethanol treatment using the liquid diet method in C57BL/6J mice. Additionally, to elucidate the relationship between the change in NR2B subunits and the expression of ethanol withdrawal signs, we investigated the change in NR2B subunit proteins in the cerebral cortex and limbic forebrain of ethanol-dependent and -withdrawn mice.

2. Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory

* Corresponding author. Department of Pharmacology, School of Pharmacy, Hoshi University 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. Tel.: +81-3-5498-5831; fax: +81-3-5498-5831.
E-mail address: suzuki@hoshi.ac.jp (T. Suzuki).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00428-3

Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture of Japan.

2.1. Animals

Male C57BL/6J mice (14–19 g) were obtained from Tokyo Animal Laboratories (Tokyo, Japan). The animals were housed at a room temperature of 22 ± 1°C with a 12-h light–dark cycle (light on 8:00 am to 8:00 pm). Food and water were available ad libitum during pre-experimental period.

2.2. Behavioural experiment

2.2.1. Development of physical dependence on ethanol

To prepare the liquid diet, 95% ethanol (Wako Pure chemical, Osaka, Japan) and sucrose (Wako Pure chemical) were mixed with a skimmed milk according to the method described by Iso (1984). For the chronic ethanol treatment, mice were individually housed and given access to a measured amount of liquid diet containing 5% (w/v) ethanol as their sole nutrient source for 5 days. Control mice were pair-fed a liquid diet in which sucrose was substituted in isocaloric quantities for ethanol. Every 24 h, body weight and liquid diet consumption were measured and the liquid diet was replaced by fresh ethanol-containing liquid diet.

2.2.2. Ethanol withdrawal

Withdrawal was induced by substituting normal liquid diet for ethanol-containing liquid diet at 10:00 am on day 6 following ethanol treatment. To quantify the intensity of the physical dependence on ethanol, withdrawal signs were rated on a scale of 0–5, modified from a scale described previously (Ritzmann and Tabakoff, 1976). The ratings were as follows: 0, little or no reaction; 1, piloerection or jerk; 2, weak tremor; 3, severe tremor; 4, handling-elicited or spontaneous clonic–tonic seizure; 5, death while in a seizure. Withdrawal signs were observed at 3, 6, 9, 12 and 48 h after the discontinuation of ethanol treatment. Mice were treated with vehicle or ifenprodil (10 mg/kg, i.p.) 30 min before every observation of withdrawal signs after the withdrawal.

2.2.3. Drugs

Ifenprodil tartrate (Gleran Pharmaceutical CO, Tokyo, Japan) was dissolved in DMSO (dimethyl sulfoxide: Kanto Chemical, Tokyo, Japan) and diluted in 5% DMSO with 9% Tween 80 (Wako Pure chemical)/saline before use.

2.3. Biochemical experiment

2.3.1. Tissue dissection and preparation

In accordance with the method described by Franklin and Paxinos (1997), the brain was removed following chronic ethanol treatment for 5 days (0 h), and at 9 and 48 h after the discontinuation of ethanol treatment for 5 days. For the control group, mice were treated with normal liquid diet. The cerebral cortex and limbic forebrain (containing the nucleus accumbens and olfactory tubercles) were then dissected on an ice-cold metal plate. The tissue was homogenized in 10 volumes of ice-cold buffer A containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 0.1 mg/ml aprotinin and 0.32 M sucrose using a Potter-Elvehjem tissue grinder with Teflon pestle. The homogenate was then centrifuged $1000 \times g$ for 10 min, and the supernatant was centrifuged at $20\,000 \times g$ for 30 min at 4°C. The pellets were then re-homogenated and re-centrifugated at $20\,000 \times g$ for 30 min at 4°C. The resulting pellets were resuspended and retained as membrane fractions.

2.3.2. Immunoblotting of NMDA receptor subunits

Detection of NMDA receptor subunits was conducted by immunoblot. Protein concentration in the samples was then assayed. An aliquot of tissue sample was diluted with an equal volume of $2\times$ electrophoresis sample buffer (Protein Gel Loading Dye-2x; AMRESCO, Solon, OH) containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol with 0.2 M dithiothreitol. Proteins (8–20 μg/lane) were separated by size on 4–20% SDS–polyacrylamide gradient gel using the buffer system and transferred to nitrocellulose membranes in Tris–glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection of NMDA receptor subunits, membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dried milk (BIO-RAD Laboratories, Hercules, CA) for 1 h at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS (NMDA ε2 (NR2B), 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) containing 5% nonfat dried milk overnight at 4°C. The membrane was then washed twice for 5 min and then twice for 10 min in Tween 20-TBS (TTBS) containing TBS and 0.05% Tween 20, followed by 2-h incubation at room temperature with horseradish peroxidase-conjugated rabbit anti-goat IgG (Southern Biotechnology Associates, Birmingham, AL) diluted 1:10 000 in TBS-containing 5% nonfat dried milk. After this incubation, the membranes were washed twice for 5 min and then three times for 10 min in TTBS. The antigen–antibody peroxidase complex was then finally detected by enhanced chemiluminesence (PIERCE, Rockford, IL) according to the manufacture's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Science, Arlington Heights, IL). Film autoradiograms were analyzed and quantified using the NIH imaging system.

2.4. Statistical analysis

Analyses of the withdrawal score were performed using Student's *T*-test. The immnoblot data were expressed as

percentage of increase (mean ± S.E.) with respect to the control group, and the statistical analysis was performed using Student's *T*-test.

3. Results

3.1. Effect of ifenprodil on the withdrawal signs induced by the termination of ethanol treatment

Groups of mice were divided into two groups; vehicle- and ifenprodil-challenged groups. Both groups were treated with 5% ethanol for 5 days. There was no significant difference in ethanol intake between two groups (data not shown). After the discontinuation of ethanol treatment, piloerection, jerk, tremor and handling-elicited convulsion were observed. The withdrawal scores of vehicle-treated mice gradually increased; the maximal score was observed at 9 h after the withdrawal. The withdrawal signs lasted at least for 12 h and had almost terminated by 48 h after the discontinuation.

The time-course of withdrawal scores in vehicle- or ifenprodil-challenged mice is shown in Fig. 1. At 6, 9 and 12 h after the withdrawal, treatment with ifenprodil (10 mg/kg, i.p.) significantly suppressed the withdrawal score as compared to the vehicle control (6 h, $P < 0.05$; 9 h, $P < 0.001$; 12 h, $P < 0.01$).

3.2. Effect of chronic ethanol treatment on the protein level of NR2B subunits

The effect of chronic ethanol treatment on the protein level of NR2B subunits is shown in Fig 2. In the cerebral cortex, there was no change in the protein level of NR2B subunits at 0, 9 and 48 h after the withdrawal. In contrast, the protein level of NR2B subunits in the limbic forebrain significantly increased by 60.9 ± 3.1% following chronic ethanol treatment (0 h, $P < 0.001$). The up-regulation lasted at least for 9 h after the withdrawal, however, the level in NR2B subunits gradually returned to the basal level within 48 h after the withdrawal.

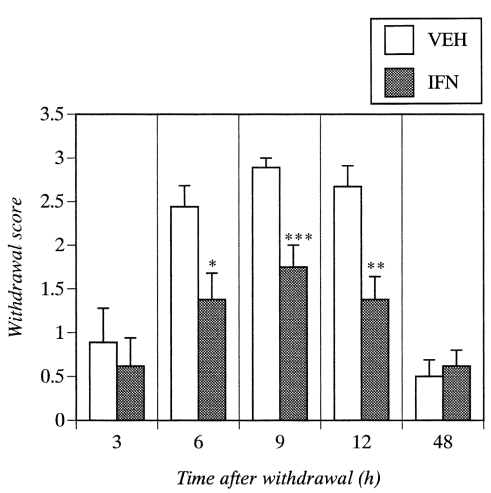

Fig. 1. Effects of ifenprodil (10 mg/kg, i.p.) on the time-course change in the mean withdrawal score after the discontinuation of chronic ethanol treatment. Mice were treated with 5% ethanol-containing liquid diet for 5 days and then injected i.p. with vehicle (VEH) or ifenprodil (IFN) 30 min before the observation at 3, 6, 9 and 12 h after the discontinuation of ethanol treatment. Each column represents the mean with S.E.M. of 8–9 mice; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. VEH-challenged group.

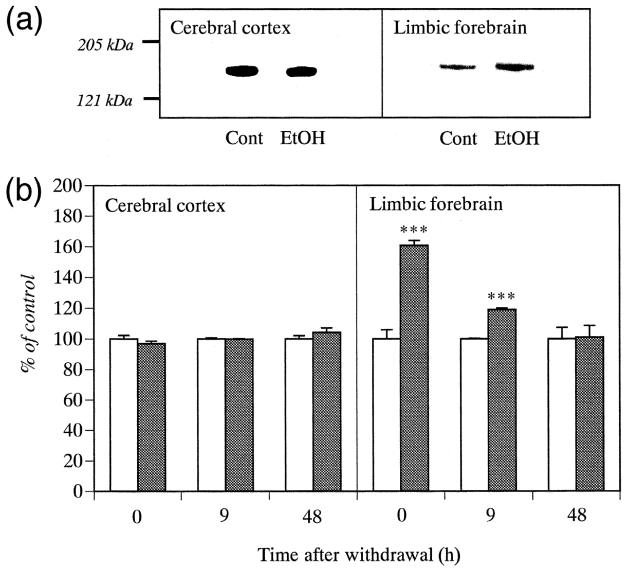

Fig. 2. Protein levels of the NR2B subunit in the cerebral cortex and limbic forebrain during ethanol withdrawal. (a) Representative Western blot of NMDA receptor NR2B subunit protein following chronic ethanol treatment (0 h) in control (Cont) and ethanol-withdrawn (EtOH) mice. (b) Changes in the protein level of the NR2B subunit in the cerebral cortex and limbic forebrain from control (open column) and ethanol-withdrawn (closed column) mice. Mice were treated with normal diet or 5% ethanol-containing liquid diet for 5 days. The membrane fraction was prepared at 0 (ethanol-dependent), 9 and 48 h after the discontinuation of ethanol treatment. Each column represents the mean with S.E.M. of 4–8 samples; $^{***}P < 0.001$ vs. control mice.

4. Discussion

In the present study, mice were treated with 5% ethanol-containing liquid diet for 5 days. After the discontinuation of ethanol treatment, withdrawal signs such as piloerection, jerk, tremor and handling-elicited convulsion were clearly observed. It has been reported that the same kinds of withdrawal signs are observed after withdrawal using different methods to develop ethanol dependence (Goldstein, 1972; Ritzmann and Tabakoff, 1976), indicating that physical dependence on ethanol can be reliably developed using the liquid diet method.

Here we found that the expression of spontaneous ethanol withdrawal signs in mice was suppressed by treatment with a selective NR2B-containing NMDA receptor antagonist, ifenprodil. Earlier reports have revealed that other NMDA receptor antagonists such as dizocilpine and

eliprodil suppressed the expression of ethanol withdrawal signs (Grant et al., 1990; Kotlinska and Liljequist, 1996). Considering the fact that ifenprodil possesses a high affinity for NR2B-containing NMDA receptors, the regulation of NR2B-containing NMDA receptors may be implicated in the expression of ethanol withdrawal.

The present study demonstrated for the first time that an up-regulation of the carboxyl-terminal of NR2B subunits in the limbic forebrain including the nucleus accumbens was clearly observed during ethanol treatment. The up-regulation of NR2B subunits gradually returned to the control level within 48 h after the discontinuation of ethanol treatment, thereby implying that the ethanol-induced changes at this stage were reversible. These results suggest the possibility that an up-regulation of the carboxyl-terminal of NR2B subunits in the limbic forebrain during chronic ethanol exposure may result from adaptive response to the inhibitory effect of acute ethanol treatment on glutamate release. Furthermore, the induction of glutamate release and the abrupt disappearance of post-synaptic inhibition induced by ethanol after the discontinuation of ethanol treatment is likely to produce ethanol withdrawal signs associated with the overexcitation of NR2B subunit-dependent post-synaptic neurotransmission, finally resulting in the reset of glutaminergic systems.

It is well-known that the mesolimbic dopaminergic neurons are originated from the ventral tegmental area (VTA) and mainly projected to the limbic forebrain including the nucleus accumbens (Kalivas, 1993). Interestingly, a dopamine D2 receptor antagonist has been shown to attenuate convulsions during ethanol withdrawal syndrome in mice (Naassila et al., 1998). These findings strongly support the hypothesis that the blockade of NR2B subunits co-localized with dopamine receptors in the nucleus accumbens may reduce the induction of ethanol-withdrawal signs following an up-regulation of NR2B subunits in this area.

It is of interest to note that the phosphorylation sites for either protein kinase C or tyrosine kinase present at the extreme carboxyl-terminal region of NR2B subunits (Mori et al., 1993; Wagner and Leonard, 1996; Logan et al., 1999). It has been also proposed that tyrosine phosphorylation of NR2B subunit is most likely to be critical for regulating NMDA system-dependent neuronal plasticity (Rosenblum et al., 1996; Rostas et al., 1996). Considering the above reports, a hypothesis may be advanced that the induction of the phosphorylation of NR2B subunits by ethanol treatment leads to such increased excitability of the glutaminergic neurotransmission following ethanol withdrawal.

In the cerebral cortex, the level of NR2B subunit proteins was not altered. The finding was not consistent with the report by Kalluri et al. (1998) that chronic ethanol treatment increased polypeptide levels of NR2B subunits in the cerebral cortex. Although it is not yet clear why the protein level of NR2B subunits in the cerebral cortex was unchanged under the present conditions, it is probable that the discrepancy results from different methodologies of developing ethanol dependence. In their studies, rats were intoxicated by intragastric intubation and compulsorily treated with a higher concentration (20%) of ethanol for a longer period (Kalluri et al., 1998). Thus, we propose here that the NR2B-containing NMDA subunit in the limbic forebrain may be primary affected by chronic ethanol treatment, resulting in initiating ethanol dependence.

In conclusion, treatment with a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil suppressed the expression of spontaneous ethanol withdrawal signs, and the NR2B subunit was markedly up-regulated in the mouse limbic forebrain, but not cerebral cortex, during ethanol treatment. These findings suggest that an up-regulation of NR2B subunits may be, at least in part, implicated in the development of physical dependence on ethanol in mice.

Acknowledgements

This work was supported in part by grants from the Ministry of Health and Welfare, and the Ministry of Education, Science, Sports and Culture of Japan to T. Suzuki. We wish to thank Ms. Hiroko Tamaki for her excellent technical assistance.

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