Chronic Ethanol-mediated Up-regulation of the N-Methyl-D-aspartate Receptor Polypeptide Subunits in Mouse Cortical Neurons in Culture* (Received for publication, February 23, 1996, and in revised form, April 2, 1996)

Paulo Follesa and Maharaj K. Ticku†
From the Department of Pharmacology, University of Texas Health Science Center, San Antonio, Texas 78284-7764

The goal of this study was to determine whether chronic ethanol-mediated up-regulation of the N-methyl-D-aspartate receptors (NMDAR) was associated with an augmentation of the NMDAR polypeptide subunits in the mammalian cortical neurons. The results show that chronic ethanol treatment produced an increase in the R1 and R2B polypeptide subunits. The R2A subunit was not expressed in these neurons. Chronic NMDAR antagonist ((+)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP)) treatment also increased the R1 and R2B polypeptide subunits. A similar increase was observed when ethanol and CPP were used in combination. Binding studies using [3H]MK-801 ([+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate), a noncompetitive NMDAR antagonist, confirmed that concomitant exposure of ethanol and CPP up-regulated the NMDAR. Our results demonstrate for the first time that chronic ethanol treatment increased the NMDA receptor polypeptide subunit synthesis and that it was associated with an increase in [3H]MK-801 binding sites.

The glutamate system is involved in the ethanol-induced tolerance, physical dependence, and withdrawal syndrome (1). In particular there is evidence for the involvement of the NMDAR1-mediated neurotransmission (2). Several studies have shown that chronic ethanol treatment, in vivo and in vitro, increased the density of [3H]MK-801 binding sites to the NMDAR complex (3–5) and the NMDAR function (5). Moreover, chronic ethanol treatment produced a differential regulation of the mRNA synthesis of the two families of subunits of the NMDAR complex in the rat brain and in mouse cultured neurons (6, 7). In addition, a study has demonstrated an increase of the R1 subunit immunoreactivity following chronic ethanol treatment in selected brain regions (8), despite no apparent change in the mRNA levels for the same subunit using a similar paradigm (6). There is no information available about the effect of chronic ethanol treatment on the other family, the R2, of polypeptides of the NMDAR complex. The two classes of subunits can be present in the same receptor conferring a different pharmacology depending upon the subtype present (9, 10).

The relationship between the increase in ethanol-mediated [3H]MK-801 binding sites, the increase in mRNA, and polypeptides of the different NMDAR subunits is unclear. In the present study, we measured the immunoreactivity of the R1, R2A, and R2B polypeptide subunits of the NMDAR complex in the cortical neurons in culture, following chronic ethanol exposure. Our experimental results show that chronic ethanol or chronic NMDAR antagonist treatment up-regulated the NMDAR subunit polypeptide levels. The up-regulation of the R1 subunit polypeptide appears to correlate with the up-regulation of the NMDAR binding (as measured by MK-801) following chronic ethanol treatment.

EXPERIMENTAL PROCEDURES

Cell Culture—Time pregnant mice (strain C57BL/6) were purchased from Harlan (Indianapolis, IN). All animals were used in accordance with institutional guidelines, and procedures were approved by the animal welfare committee. Mouse cortical neurons in culture were prepared as described previously (7, 11). Briefly cerebral hemispheres were dissected from 14–15-day-old fetuses, and cells were dissociated by trituration using a Pasteur pipette. Dissociated cells were counted under a microscope using a hemocytometer, and the trypan blue stain method was used to determine the number of viable cells. Cells were resuspended in the culture medium to have 2 × 10⁶ cells/ml, and 12 ml of this cell suspension were used for each poly-l-lysine-coated 75-cm² plastic flask. The cells were grown in minimum essential medium (MEM) containing 33.3 mM glucose, 26.2 mM NaHCO₃, 100 µM l-glutamate, 10% fetal bovine serum, and 10% horse serum (MEM 10/10) in an incubator with 95% O₂, 5 % CO₂ at 37°C. On the 2nd day in culture, a mixture of 5-fluoro-2′-deoxyuridine and uridine at final concentration of 10 µM was added to the culture medium (MEM 5/5) to inhibit cell proliferation (12). The cultures contain ∼92% neurons, so the contribution of glial cells, if any, will be minimal. Furthermore, NMDAR receptor subunits mRNAs are not expressed by glial cells (13).

Chronic exposure of the cells to ethanol (McCormick, Weston, MO) and/or the NMDAR antagonist ((+)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Research Biochemicals International, Natick, MA) was initiated on the 3rd day in culture and lasted for 5 days. MEM 5/5 containing freshly prepared drugs (at the indicated concentration) was replaced every 24 h. All drugs were dissolved in MEM. On the 7th day in culture, cells were harvested for membrane preparation.

Membrane Preparation and Binding Studies—Membranes were washed and cells were homogenized in EDTA buffer (5 mM K-EDTA, pH 7.0) with a Polytron homogenizer. Membrane homogenates were centrifuged (100,000 × g, 30 min), and pellets were resuspended in 20 ml of the same buffer, incubated for 30 min at 37°C, and then centrifuged (100,000 × g, 30 min). Pellets were resuspended in EDTA buffer, and the washing procedure, including the 30-min incubation, was repeated two more times. The pellets were resuspended in HEPES buffer (20 mM K-HEPES, 1 mM K-EDTA, pH 7.0), centrifuged, and stored at −80°C. On the day of the assay, membranes were thawed, diluted in assay buffer, centrifuged (100,000 × g, 30 min), and resuspended in assay buffer. The binding assay with [3H]MK-801 (4 nM) was carried out using 150–250 µM membrane protein/tube/min. Binding assays were performed in the presence of 100 µM glycine, 100 µM spermine, and 0.32 mM MgCl₂. Triplicate samples were incubated at 32°C for 3 h. Nonspecific binding was determined in the presence of 10 µM (+)-MK-801. Assays were terminated by rapid filtration as described previously (14). Values were expressed in fmol/mg protein.

Electrophoresis and Immunoblot of NMDA—Membrane preparation used for the binding studies was also utilized for the
immunoblot experiments. Membrane homogenates (25 μg of protein; 15 μg of protein for mouse brain preparation) were separated by 8.5% SDS-polyacrylamide gel electrophoresis (15). Two gels were run in parallel. One was stained with Coomassie Blue and used to normalize the data and ensure equal loading on each well of the gel; the other was used for the immunoblot. Proteins were transferred on a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). The membrane was washed with a blocking solution containing 20 mM Tris base, 137 mM sodium chloride, 5% milk powder, and 0.05% Tween 20 (TBS-T). The blot was then incubated with either one of the following: rabbit anti-NMDAR R1 polyclonal antibody affinity purified (AB1516, selective for all four splice variants), 0.5 μg/ml; rabbit anti-R2A serum (AB1555, selective for the R2A subunit), dilution 1:5,000; rabbit anti-R2B serum (AB1557, selective for R2B subunit), dilution 1:5,000 (the three antibodies were purchased from Chemicon, Temecula, CA). The blot was washed several times with TBS-T and then incubated with the secondary antibody, peroxidase-conjugated anti-rabbit IgG (dilution 1:10,000) (Amersham Corp.). Specific bands were visualized on a film (Kodak X-omat LS; Kodak, Rochester, NY) using the ECL detection system (Amersham Corp.). All blots were stripped and reprobed once with each antibody. To check for efficacy of protein transfer from the gel to and to match Coomassie-stained bands with immunoreactive proteins, reversible staining of the membrane after transfer with Ponceau S was done to ensure homogeneity of transfer. The relative changes in the NMDAR polypeptide levels were evaluated by measuring the intensity of the immunoreactive bands using the NIH IMAGE system (11,16). The data were normalized to the amount of protein loaded in each lane of the Coomassie Blue stained gel that was run in parallel by measuring both the major band at 58 kDa and the intensity of the whole lane. The amount of protein loaded on the gel gave immunoreactive bands with density in the linear range of intensity. The data were expressed as percentage of increase with respect to the control group ± S.E., and the statistical analysis was performed using analysis of variance and Sheffe’s test.

RESULTS

Chronic ethanol treatment did not alter the morphological characteristics of the cortical neurons, protein content, or the resting intracellular (Ca$^{2+}$) concentrations (5,2). The effect of chronic ethanol treatment (75 mM; 5 days) on NMDAR polypeptide subunit expression of cortical neurons in culture is shown in Fig. 1. Immunoblot studies showed that chronic ethanol treatment increased the levels of the R1 polypeptide subunit by 27% with respect to the control group (Table I). The R2 polypeptide subunit levels were also increased (63%, Table I). The R2A polypeptide subunit was not expressed in the cortical neurons in culture at this stage of development (Fig. 1A). To ensure that the antibody against the R2A polypeptide subunit was working, we loaded on the same gel 15 μg of membrane proteins from mouse brain that showed an immunoreactive band of 165 kDa (Fig. 1A). This demonstrates the absence of the R2A polypeptide subunit in the cortical neurons in culture at this stage of development.

Chronic treatment with the NMDAR antagonist CPP (10 μM; 5 days) also up-regulated the expression of the R1 and R2 polypeptide subunits of the NMDAR (Table I and Fig. 1, B and C). The CPP-mediated increase in the R1 and R2 polypeptide subunits levels was larger than the ethanol-mediated increase (Table I). Concomitant exposure of ethanol and CPP showed the same increase as observed with CPP alone (Table I). The statistical analysis indicated that there was not a significant difference between CPP and ethanol + CPP treatments for both the R1 and R2 polypeptide subunits (p = 0.743 and p = 0.999, respectively, Sheffe’s test). In addition, we used the same membrane preparation utilized for the immunoblot studies to measure the [3H]MK-801 binding. We found that chronic ethanol treatment increased (20%) the number of binding sites for this radioligand (Fig. 2 and Table II). A larger increase in [3H]MK-801 binding sites was observed in cells treated with CPP or ethanol + CPP (Table II and Fig. 2). The increase in [3H]MK-801 binding sites was observed in cells treated with CPP or ethanol + CPP (Table II and Fig. 2).

DISCUSSION

Electrophysiological and biochemical studies demonstrate that ethanol acutely inhibits the NMDAR function (1,2). In contrast, chronic ethanol treatment increases the number of NMDARs and their function in vivo and in vitro (3,5). These observations suggest that the NMDAR-mediated glutamate neurotransmission may be involved in the ethanol dependence of ethanol withdrawal.
In summary, these results demonstrate for the first time that chronic ethanol treatment increased both classes of polypeptide subunits, the R1 and the R2, of the NMDAR. Taken together with our previous observations, these data suggest that the ethanol- and CPP-mediated increase in mRNA and polypeptide could be controlled independently from each other.

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