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Chronic Ethanol Increases *N*-Methyl-D-Aspartate-Stimulated Nitric Oxide Formation but Not Receptor Density in Cultured Cortical Neurons

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SUMMARY

The effects of prolonged ethanol exposure on excitatory amino acid receptor stimulated nitric oxide (NO) formation were examined in primary rat cortical neuronal cultures. Chronic ethanol (4 days, 100 mM) potentiated *N*-methyl-D-aspartate (NMDA)-stimulated NO formation as determined by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline. In contrast, chronic ethanol had no effect on NO formation stimulated by kainate, α-amino-3-hydroxy-5-methyl-4-isoxalonepropionic acid, or the calcium ionophore ionomycin. Potassium chloride-stimulated NO formation was also enhanced by chronic ethanol treatment, but this effect was not seen in the presence of the ionotropic glutamate receptor antagonists MK-801 and 6-cyano-7-nitroquinoxaline-2,3-dione. Immunoblot analysis of expression of NR1, NR2A, and NR2B receptor subunits showed no difference between control and chronic ethanol-treated cultures. In support of this apparent lack of change in receptor density, there was no difference in the specific binding of ^{125}I-MK-801 between control and chronic ethanol-treated groups. These results demonstrate that prolonged ethanol exposure selectively enhanced NMDA receptor-stimulated NO formation, which may play an important role in alcohol dependence, withdrawal, and alcohol-associated brain damage. These results also suggest that chronic ethanol-induced increases in NMDA receptor function may not be due to a simple increase in the number of NMDA receptors or change in NMDA receptor subunit composition but may instead reflect more complicated and subtle changes.

NO is a short-lived gas that participates in diverse physiological processes (1). It is formed endogenously in a number of tissues from L-arginine by NOS, an enzyme that exists as several different isoforms. Although the exact functions of NO in the central nervous system are not yet clear, it seems to be a novel type of neurotransmitter that exhibits both physiological and pathological actions. Activation of neuronal NOS is calcium dependent, and in brain, it seems to be primarily coupled to activation of glutamate receptors (2). Glutamate is the major excitatory neurotransmitter, and it acts through two classes of membrane receptors that include G-protein linked metabotropic receptors and three subtypes of ionotropic receptors named after their selective agonists: NMDA, AMPA, and kainate receptors. NMDA receptors are highly permeable to calcium and play a particularly important role in a number of physiological processes, including neuronal development, synaptic plasticity, and learning and memory (3). In addition to physiological processes, excessive or inappropriate stimulation of NMDA receptors can trigger a series of events that result in delayed neuronal cell death. This process, termed excitotoxicity, has been implicated in a number of acute neuropathological conditions (e.g., stroke) as well as several chronic neurodegenerative conditions (e.g., Huntington's disease). Recent evidence suggests that NO may be an important mediator of both the physiological and pathological actions of NMDA receptors. For example, NO is thought to be a retrograde messenger in response to activation of NMDA receptors during long term potentiation and to play a role in NMDA neurotoxicity (4).

Accumulating evidence suggests that the behavioral, neurophysiological, and pathological effects of alcohol are mediated in large part through the glutamatergic system, and

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; NR, *N*-methyl-D-aspartate receptor subunit; PBST, phosphate-buffered saline/Tween 20; SDS, sodium dodecyl sulfate; PDHS, plasma-derived horse serum.

NMDA receptors seem to be particularly important sites of action of ethanol. A number of electrophysiological as well as biochemical studies have clearly demonstrated that ethanol can inhibit NMDA receptor currents and NMDA receptor-mediated responses (see Ref. 5 for a complete review). In contrast to the inhibitory effects of acute ethanol, chronic ethanol exposure can enhance NMDA receptor function (6, 7). Enhanced NMDA receptor-mediated glutamatergic neurotransmission has been implicated in the ethanol withdrawal syndrome and may play a role in ethanol-related brain damage (8, 9). Increased NO production would be expected to contribute to enhanced excitotoxicity and perhaps other changes associated with chronic ethanol.

A number of recent studies have suggested that NO may play an important role in the acute and chronic effects of ethanol. NOS inhibitors have been reported to block the development of rapid tolerance to the motor incoordinating effects of alcohol (10, 11) and to block alcohol-induced suppression of testosterone secretion (12). Similarly, administration of NOS inhibitors during alcohol withdrawal can decrease the severity of withdrawal signs and symptoms (13). Neuronal formation of NO in brain is primarily linked to activation of glutamate receptors, and involvement of NO in the actions of alcohol is probably linked to changes in glutamate receptor function. We have previously shown that NMDA stimulation of NO formation in cultured cortical neurons is reduced in the presence of acute ethanol (2). In the current study, we investigated the effects of chronic ethanol exposure on both neuronal NO formation and NMDA receptor expression in primary neuronal cultures.

Experimental Procedures

Materials. Female Sprague-Dawley rats were obtained from Charles River Farms (Wilmington, MA) and housed and bred in our animal facility. DMEM and amphotericin B (Fungizone) was purchased from GIBCO (Grand Island, NY). PDHS was purchased from Central Biomedia (Irwin, MO), and trypsin was obtained from Worthington Biochemicals (Freehold, NJ). Penicillin and streptomycin were purchased from Pfizer Inc. (New York, NY). [^{3}H]L-Arginine was purchased from Amersham (Arlington Heights, IL), and Dowex (AG50WX-8, 100–200 mesh) was from BioRad (Melville, NY). CNQX was purchased from Tocris Neuramin (Essex, UK), and MK-801 and AMPA were from Research Biochemicals (Natick, MA). Antineuronal-NOS (brain NOS) was purchased from Transduction Laboratories (Lexington, KY), anti-NR1 was from PharMingen (San Diego, CA), and anti-NR2A and anti-NR2B were from Calbiochem (San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit and horse anti-mouse immunoglobulins G were purchased from Vector Laboratories (Burlingame, CA) and Southern Biotechnology Associates (Birmingham, AL), respectively.

Preparation of neuronal cultures. Cerebral cortical cultures were prepared from 1-day-old rat pups exactly as previously described (2). In brief, brains were removed from 1-day-old pups and placed in an isotonic salt solution containing 100 units of penicillin G/100 μg of streptomycin/0.25 μg of amphotericin B (Fungizone)/ml, pH 7.4. Blood vessels and pia mater were removed, and the tissue was chopped into ~2-mm chunks. The brain pieces were then suspended in 25 ml of 0.25% trypsin (w/v) in isotonic salt solution, pH 7.4, and placed in a shaking water bath for 10 min at 37° to dissociate the cells. The dissociated cell suspension was then removed and combined with 10 ml of DMEM containing 10% (PDHS), and the undissociated chunks were mixed with 160 μg of DNase 1 and triturated until the cells had dissociated. The dissociated cell suspensions were combined and centrifuged at 1000 $\times$ *g* for 10 min, and the resulting pellet was washed with 50 ml DMEM containing 10% PDHS. Cells were resuspended in DMEM containing 10% PDHS and plated onto poly-L-lysine-precoated culture dishes at a density of 4 $\times$ 10^6 cells/35-mm dish and incubated at 37° in a humidified incubator with 5% CO_2/95% air. On day 3, cells were treated with 10 μM β-cytosine arabinoside in DMEM containing 10% PDHS. After 2 days of treatment with β-cytosine arabinoside, culture medium was replaced with DMEM containing 10% PDHS, and cultures were grown for an additional 7–9 days before being used in experiments. At this time, cultures consisted of ~90% neurons as determined by immunofluorescent staining with antineurofilament NE14 (14). Cultures appeared as many phase-bright cells with characteristic neuronal morphology overlying a small number of flat phase-dark cells that had typical astroglial morphology. Each experimental number (*n*) represents data obtained from neuronal cultures prepared from separate litters of pups.

Measurement of NOS activity. NOS activity was determined by measuring the formation of [^{3}H]L-citrulline from cells preloaded with [^{3}H]L-arginine as previously described (2). Cortical cultures were washed twice with 1 ml of HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 100 nM glycine 15 mM glucose, 25 mM HEPES, pH 7.4) and incubated for 10 min in 1 ml of HEPES buffer. [^{3}H]L-Arginine (3 μCi/ml) was then added to each culture dish and allowed to incubate for 5 min followed by addition of the indicated agonist. In some experiments, ethanol, Mg^{2+}, or receptor antagonists were added at the start of the 10-min preincubation period. After a 5-min agonist exposure, incubations were terminated by the rapid aspiration of the incubation buffer, two washes with 1 ml of ice-cold stop buffer (118 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 24.8 mM $NaHCO_3$, 4 mM EDTA, 5 mM L-arginine), and the addition of 1 ml of 0.3 M $HClO_4$. After 10 min, the cell extract was neutralized with 3.0 M K_2CO_3, and 50-μl aliquots of the tissue extract were taken for determination of total uptake of [^{3}H]L-arginine by liquid scintillation counting. To determine formation of [^{3}H]citrulline from [^{3}H]arginine, a 500-μl aliquot of the neutralized extract was applied to columns containing 2 ml of a slurry of Dowex AG50WX 8 (Na^+ form) and H_2O (1:1) and washed three times with 2 ml of H_2O, and the eluant (6 ml) was collected into scintillation vials. Thin layer chromatography was used to verify that the radioactivity in the eluant was [^{3}H]citrulline. Ten milliliters of scintillation cocktail was then added, and the amount of radioactivity recovered in the eluant was quantified by liquid scintillation counting. [^{3}H]Citrulline formation was expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells.

Chronic ethanol exposure and preparation of membranes. After 8 days in culture, some dishes were treated with 100 mM ethanol and placed in a Nalgene Zip-Lock bag along with a beaker containing 500 ml of 120 mM ethanol, which maintains the ethanol concentration in the dishes at 100 mM. The bags were filled with 5% CO_2/95% air and returned to the incubator for an additional 96 hr. Control dishes were treated identically but were not exposed to ethanol. For preparation of membranes from cultured neurons, cells from control and chronic ethanol dishes were scraped into ice-cold 5 mM K^+-EDTA, pH 7.0, buffer (1 ml/dish). For preparation of membranes from 4- and 26-day-old rat pups, brains were dissected on ice and placed in K^+-EDTA buffer. The cell suspension or dissected brain region was homogenized using a Tekmar Tissumizer for 20 sec and centrifuged at 48,000 $\times$ *g* for 30 min at 4°. The resulting pellet was washed twice and resuspended in K^+-EDTA buffer followed by centrifugation. Before the final spin, the membrane suspension was separated into aliquots, and the resulting pellets were kept at −70° until their use.

Gel electrophoresis and immunoblotting. For analysis of ionotropic glutamate receptor expression, membrane pellets were resuspended in 2% SDS, probe sonicated for 20 sec and boiled for 5 min to denature the proteins. For analysis of neuronal-NOS expression, solubilized proteins were prepared from intact cells instead of isolated membranes by the direct addition of 2% SDS to the culture

dishes. A small aliquot was removed for determination of protein concentration by the bicinchoninic acid assay (Pierce Chemical, Rockford, IL), and a larger aliquot was diluted with an equal volume of 2× electrophoresis sample buffer [final concentration, 1 × 50 mM Tris·HCl, pH 6.7, 4% glycerol (w/v), 4% SDS, 1% 2-mercaptoethanol, and 0.02 mg/ml bromphenol blue]. The 2 × sample buffer contained 6% SDS to provide the final SDS concentration of 4%. Proteins were separated by size on a 7.5% (NR1) or 6% (NR2A and NR2B) SDS-polyacrylamide gel using the buffer system of Laemmli (15) and transferred to polyvinylidene difluoride membranes in Towbin-SDS transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS). After transfer, the blot was washed with PBST and stored overnight in PBST at 4°. For immunoblot detection of NMDA receptor subunits, membranes were blocked in PBST containing 5% nonfat dry milk (Carnation) and 1% bovine serum albumin for 1 hr at room temperature with agitation. The membrane was washed once with PBST and incubated with primary antibody diluted (neuronal NOS, 1:250; NR1, 1:2000; NR2A and NR2B, 1:500) in PBST containing 0.5% nonfat dried milk and 0.1% bovine serum albumin for 1 hr. The membrane was then washed once for 5 min, once for 15 min, and then twice for 5 min in PBST followed by a 1-hr incubation with agitation at room temperature with horseradish peroxidase-conjugated horse anti-mouse (NR1) or goat anti-rabbit (NR2A and NR2B) immunoglobulin G diluted 1:2000 and 1:1000, respectively, in PBST containing 0.5% nonfat dried milk and 0.1% bovine serum albumin . After this incubation, the membranes were washed as above, and the antigen/antibody/peroxidase complex was detected by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm. Membranes were immediately placed in a BioRad exposure cassette and read the following day. Film autoradiograms and exposure cassettes were analyzed and quantified by computer-assisted densitometry and phosphoimaging, respectively, using a BioRad Molecular Imaging System. Protein concentration curves in which increasing amounts of protein (5–40 μg) was loaded onto the gel showed the intensity of the bands to be linear (NR1, R^2 = 0.977; R2A, R^2 = 0.962). Thus, 20 μg of protein was used for immunoblot analysis of NMDA receptor subunits.

^{125}I-MK-801 binding. Binding assays with ^{125}I-MK-801 were performed as described by Williams *et al.* (16). In brief, membranes were thawed on the day of assay and diluted into HEPES assay buffer (1 mM K-EDTA; 20 mM HEPES, pH 7.0), homogenized using a Tekmar Tissumizer, and centrifuged at 48,000 × *g* for 60 min at 4°, and the resulting pellet was resuspended in HEPES assay buffer. Binding assays (3 hr at 37°) were performed using 15 μg of membrane protein/tube in the presence of a saturating concentration (1.5 nM) of ^{125}I-MK-801 (2200 Ci/mmol), 100 μM glutamate, 100 μM glycine, and 30 μM spermine. Saturation binding curves were carried out using seven different concentrations of ^{125}I-MK-801 (0.0625–3.0 nM), and nonspecific binding was determined in the presence of 10 μM unlabeled (+)-MK-801 (~20–30% of total binding). Assays were terminated by rapid filtration and washing with ice-cold HEPES buffer using a Brandel Cell Harvester (Montreal, Quebec, Canada).

Results

Fig. 1 shows the effects of ionotropic glutamate receptor agonists and antagonists on NOS activity in cultured cerebral cortical neurons. NMDA (50 μM) caused the largest increase in NOS activity, which was approximately twice that of kainate (50 μM) and AMPA (50 μM). NMDA stimulation of NOS activity was unchanged in the presence of the AMPA/kainate antagonist CNQX (10 μM) but completely inhibited by the NMDA antagonist MK-801 (10 μM). In contrast, MK-801 had no effect on NOS activity stimulated by either kainate or AMPA, whereas the kainate response was completely blocked by 10 μM CNQX. AMPA stimulation was

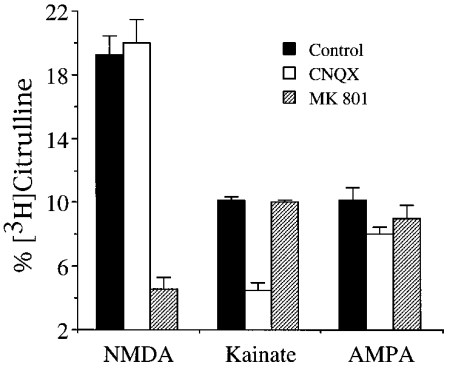

Fig. 1. Effects of ionotropic glutamate receptor agonists and antagonists on stimulation of NOS activity in primary cultures of rat cortical neurons. Cultures were preincubated with [^{3}H]arginine followed by a 5-min exposure to a 50 μM concentration of the indicated agonists in the presence and absence of 10 μM MK-801 or 10 μM CNQX. NO formation was assayed by measuring the formation of [^{3}H]citrulline from [^{3}H]arginine and is expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells. Values represent mean ± standard error (n = 3).

not significantly reduced by 10 μM CNQX but completely inhibited by 100 μM CNQX (not shown; basal, 5.2 ± 0.5%; AMPA + 100 μM CNQX, 6.2 ± 2.3%; n = 2). Thus, consistent with previous studies, all three subtypes of ionotropic glutamate receptors can couple to activation of NOS in cultured cerebral cortical neurons.

We have shown previously that acute ethanol can inhibit NMDA-stimulation of NOS activity in primary cultures of cerebral cortical neurons (2). The effects of chronic ethanol exposure on stimulation of NOS activity are shown in Fig. 2. Incubation of cultures with ethanol (100 mM) for 4 days significantly enhanced NMDA (10 μM)-stimulated NOS activity by ~28% but had no effect on stimulation by either kainate (100 μM) or AMPA (100 μM). In the case of kainate and AMPA, MK-801 was included to prevent activation of NMDA receptors that was sometimes observed at these

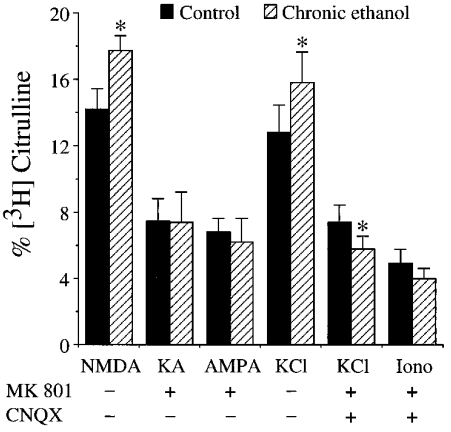

Fig. 2. Effects of chronic ethanol exposure on agonist stimulation of NOS activity in cortical neuronal cultures. Neuronal cultures were exposed to 100 mM ethanol for 4 days before the experiments. After the washout of ethanol, cultures were preincubated with [^{3}H]arginine followed by a 5-min exposure to the indicated agonists [10 μM NMDA, 30 mM KCl, 100 μM kainate (*KA*), 100 μM AMPA, 1 μM ionomycin (*Iono*)] in the presence and absence of 10 μM MK-801 and 10 μM CNQX. [^{3}H]Citrulline formation is expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells. *, $p < 0.05$, significant difference from respective control value (n = 5).

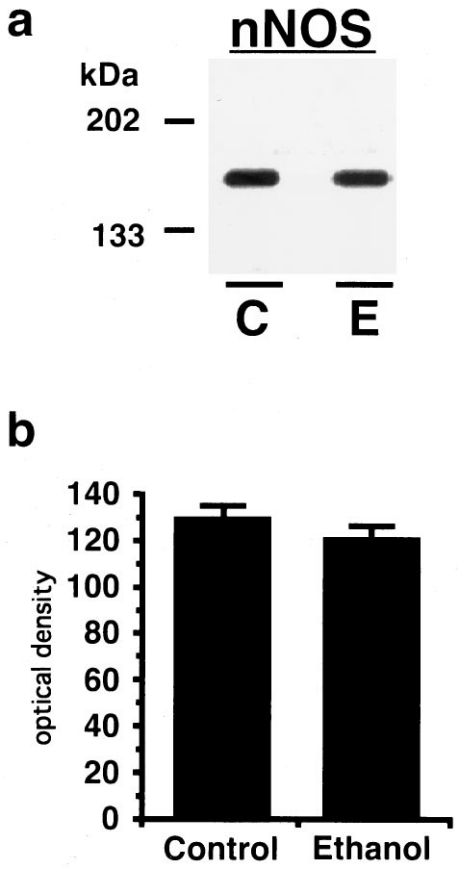

Fig. 3. Effect of chronic ethanol exposure on neuronal NOS (*nNOS*) expression in primary cortical cultures. Solubilized proteins (whole-cell) were prepared from control and sister cultures exposed to 100 mM ethanol for 4 days. Relative neuronal NOS expression was determined by immunoblot analysis using a neuronal NOS antibody and detected by enhanced chemiluminescence. A, Representative immunoblot showing the ~150-kDa band recognized by anti-neuronal NOS in (*C*) control and (*E*) chronic ethanol-exposed cultures. B, Quantification of neuronal NOS immunoreactivity by computer-assisted densitometry. Values represent mean ± standard error ($n = 4$).

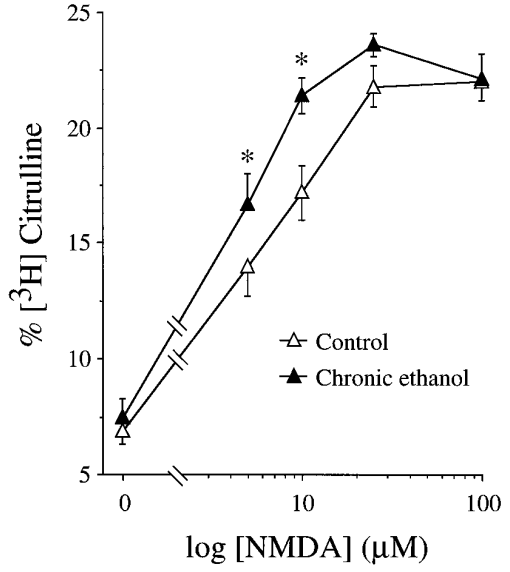

Fig. 4. Effect of chronic ethanol exposure on the NMDA concentration-response curve for stimulation of NOS activity in cortical neurons. Neuronal cultures were exposed to 100 mM ethanol for 4 days before the experiments. After this, cultures were preincubated with [^{3}H]arginine followed by a 5-min exposure to the indicated concentrations of NMDA. [^{3}H]Citrulline formation is expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells. *, $p < 0.05$, significant difference from respective control value ($n = 5$).

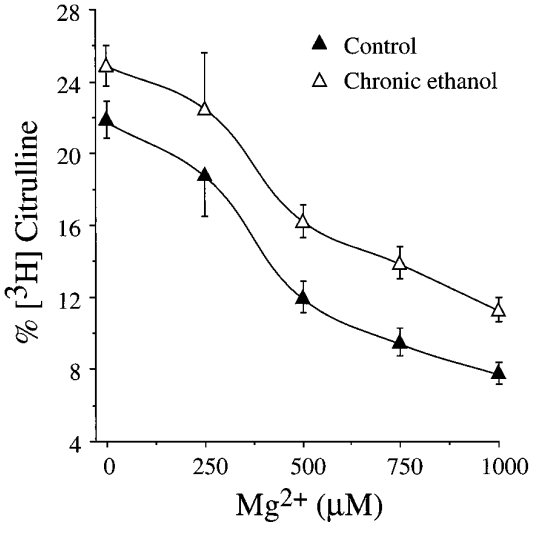

Fig. 5. Magnesium concentration response for inhibition of NMDA stimulation of NOS activity in control and chronic ethanol-exposed neuronal cultures. Cultures were incubated with 100 mM ethanol for 4 days before their use in experiments. Cultures were then preincubated with [^{3}H]arginine followed by a 5-min exposure to 10 μM NMDA in the presence and absence of the indicated concentrations of Mg^{2+}. [^{3}H]Citrulline formation is expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells ($n = 3$).

higher agonist concentrations (most likely via stimulation of glutamate release). Chronic ethanol also increased KCl-stimulated NOS activity by ~20%. However, the addition of MK-801 and CNQX reduced KCl stimulation of NOS activity by ~50% and reversed the potentiating effect of chronic ethanol, indicating that the chronic ethanol-induced increase observed with KCl resulted from stimulation of NMDA receptors secondary to KCl-induced glutamate release. In the presence of MK-801 and CNQX, there was a slight, but significant, decrease in KCl-stimulated NOS activity in chronic ethanol-treated cells. In addition, neither basal nor calcium ionophore-stimulated NOS activity was altered by chronic ethanol. Although chronic ethanol-exposed cultures showed reduced total uptake of [^{3}H]arginine (control, 327, 527 ± 8, 569 dpm/dish; 289,069 ± 6, 572 dpm/dish; $n = 10$), expression of [^{3}H]citrulline as percent conversion from total [^{3}H]arginine taken up into the cells normalized for differences in uptake. It is unlikely that reduced [^{3}H]arginine uptake contributed to the chronic ethanol enhancement of NMDA-stimulated NOS activity because stimulation of NOS activity by a variety of other agents was not altered. The differential enhancing effect of chronic ethanol on NMDA versus other NOS-activating agents also suggests that the levels of NOS protein were not altered by chronic ethanol exposure. This was confirmed by immunoblot analysis of neuronal NOS expression. A single protein band with a relative mass of ~150 kDa was detected in whole-cell lysates in both control and chronic ethanol-treated cultures (Fig. 3A), and quantification of band intensity revealed no change in neuronal NOS expression after chronic ethanol exposure

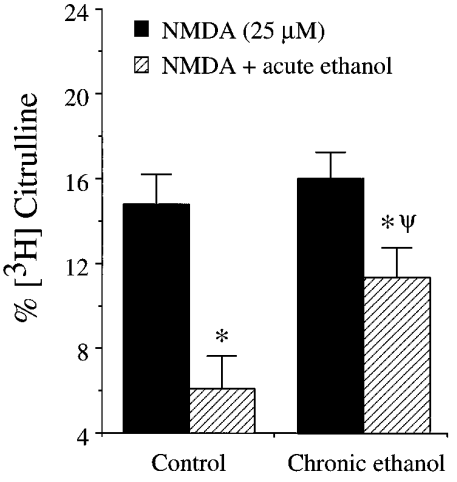

Fig. 6. NMDA stimulation of NOS activity in the presence and absence of acute ethanol in control and chronic ethanol-exposed neuronal cultures. Cultures were exposed to 100 mM ethanol for 4 days before the experiments and subsequently preincubated with [^{3}H]arginine in the presence and absence of 100 mM ethanol followed by a 5-min exposure to NMDA. [^{3}H]Citrulline formation is expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells. $*$, $p < 0.05$, significant inhibition by acute ethanol; ψ, $p < 0.05$, significant increase from respective control value ($n = 6$).

(Fig. 3B). Taken together, these data show that chronic ethanol exposure selectively potentiated NMDA-stimulated NOS activity. This effect seems to be due to enhanced NMDA receptor function and not to a change in the level of expression or calcium sensitivity of NOS.

A series of experiments were conducted to further characterize the potentiating effect of chronic ethanol on NMDA stimulation of NOS activity. Fig. 4 shows the concentration-response curve for NMDA in control and chronic ethanol-treated cultures. NMDA caused a concentration-dependent increase in NOS activity that plateaued at 25 μM NMDA with no further increase at 100 μM NMDA. Chronic ethanol-exposed cultures showed a significant enhancement in the NMDA response compared with control cultures. This increase was observed only at submaximal NMDA concentrations (5 and 10 μM) with no further increase at maximal NMDA concentrations ($\geq$25 μM). In a previous study, we reported that the inhibitory effect of acute ethanol on NMDA-stimulated NOS activity was dependent on the presence of Mg^{2+} and increased as the Mg^{2+} concentration increased. Fig. 5 shows that in the presence of 10 μM NMDA, Mg^{2+} caused a concentration-dependent inhibition of the NMDA response in both control and chronic ethanol-treated groups. At all concentrations of Mg^{2+} tested, chronic ethanol enhanced the NMDA response, resulting in a parallel upward shift in the concentration-response curve. Thus, chronic ethanol did not seem to change the Mg^{2+} sensitivity of the NMDA response. Fig. 6 shows NMDA stimulation of NOS activity in the presence and absence of ethanol in control and chronic ethanol-treated cultures. In agreement with our previous observations (2), ethanol significantly reduced NMDA (25 μM)-stimulated NOS activity in control cultures in the presence of 1 mM Mg^{2+}. Interestingly, the inhibitory effect of ethanol decreased from ~60% in controls to ~28% in chronic ethanol-treated cultures.

The chronic ethanol-induced increase in NMDA-stimulated NO formation could reflect an increase in the density of NMDA receptors. To examine this possibility, we measured the specific binding of the noncompetitive NMDA antagonist ^{125}I-MK-801. Surprisingly, the specific binding of ^{125}I-MK-801 did not differ between membranes isolated from control cultures and those from chronic ethanol-treated cultures (Fig. 7a). As a separate and independent measure of NMDA receptor density, immunoblotting procedures with NMDA receptor subunit antibodies were performed on the same membranes used for binding studies. Initial experiments concentrated on expression of the NR1 subunit because it is thought to be an essential subunit of the hetero-oligomeric NMDA receptor complex. Immunoblot analysis of NR1 subunit expression in control and chronic ethanol-treated membranes is shown in Fig. 7, b and c. The anti-NR1 antibody used recognizes all known splice forms of the NR1 subunit. A single band with a relative mass of ~116 kDa was detected in membranes from both control and ethanol-treated cultures

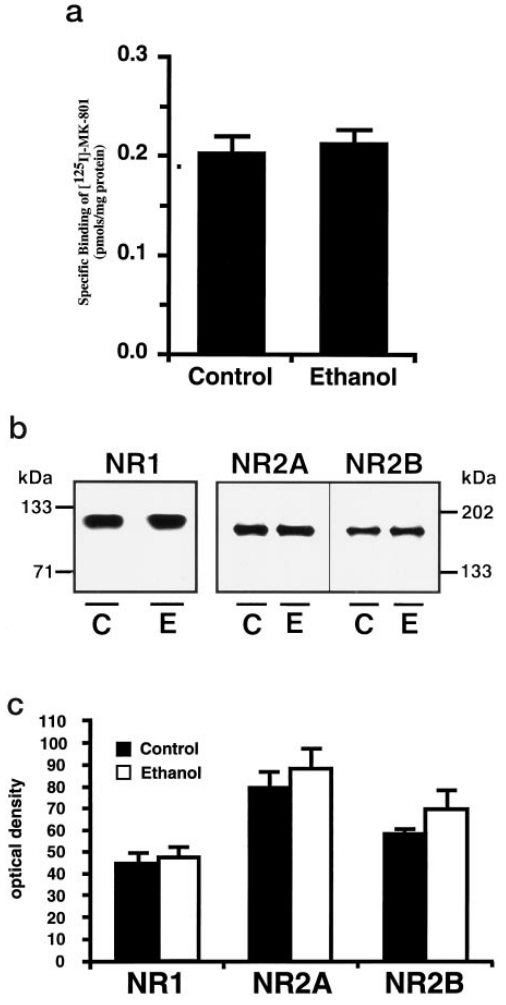

Fig. 7. Effect of chronic ethanol exposure on NMDA receptor binding density and subunit expression in neuronal cultures. a, Specific binding of ^{125}I-MK-801 in membranes isolated from control and sister cultures exposed to 100 mM ethanol for 4 days before isolation. Values are mean $\pm$ standard error ($n = 11$). b, Representative immunoblot showing the ~116-kDa band recognized by anti-NR1 and the ~175-kDa band recognized by (*left*) anti-NR2A and (*right*) anti-NR2B in membranes isolated from (*C*) control and (*E*) chronic ethanol-exposed cultures. c, Quantification of NR1, NR2A, and NR2B immunoreactivity by computer-assisted densitometry. Values represent mean $\pm$ standard error (NR1, $n = 10$; NR2A and NR2B, $n = 6$).

(Fig. 7b). The size of this protein is similar to that reported previously for the NR1 protein. In agreement with a lack of change in ^{125}I-MK-801 binding (Fig. 7a), quantification of band intensity by densitometry revealed no change in NR1 subunit expression after chronic ethanol exposure (Fig. 7c). Taken together, these observations suggest that chronic ethanol exposure did not increase the density of the hetero-oligomeric NMDA receptor complex.

The results of heterologous coexpression studies have suggested that although the NR1 subunit is an essential component of the NMDA receptor, functional diversity is conferred by differential incorporation of various NR2 subunits, primarily NR2A and NR2B. In the current study, it is possible that the enhancement of NMDA receptor function, as demonstrated by an increase in NMDA-stimulated NOS activity, is a result of a change in the NR2 subunits and not the NR1, as indicated above. To examine this possibility, immunoblot analysis using NR2A and NR2B subunit-specific antibodies was carried out on control and chronic ethanol-treated membranes. Both anti-NR2A and anti-NR2B recognized a band of ~175 kDa, which is the same as that previously reported for these subunits (Fig. 7b). In a separate experiment, we examined the postnatal developmental profile of the NR2A and NR2B subunits in membranes isolated from rat cortex and cerebellum (Fig. 8). Both NR2A and NR2B subunits in the cortex were expressed at relatively low levels at postnatal day 4 and showed a much greater level of expression at postnatal day 26. In the cerebellum, NR2A expression showed a similar low level of expression at postnatal day 4 that was also increased at day 26. In contrast, NR2B expression in the cerebellum was relatively high at postnatal day 4 and was reduced at postnatal day 26. Thus, the expression of NR2A and NR2B subunits in these two brain regions showed a differential change. This observation is in close agreement with those reported by Wang *et al.* (17) and validates our immunoblotting procedures for determining changes in NR2 subunit expression. As shown in Fig. 7, b and c, immunoreactivity of NR2A and NR2B was not significantly different in membranes isolated from control versus chronic ethanol-treated cultures. Thus, as was the case with the NR1 subunit, chronic ethanol exposure did not alter the expression of either the NR2A or NR2B subunits of the NMDA receptor.

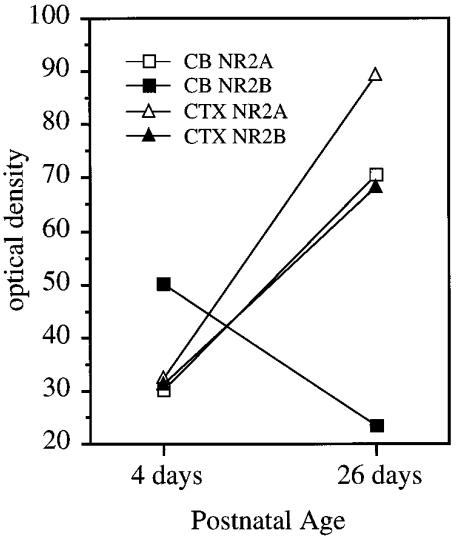

Fig. 8. Postnatal development of NR2A and NR2B immunoreactivity in rat cerebral cortex and cerebellum. Membranes were isolated from rat cerebral cortex (*CTX*) and cerebellum (*CB*) at postnatal day 4 and 26. Results of NR2A and NR2B immunoblots were analyzed by computer-assisted densitometry and represent the density values obtained from a single immunoblot.

Discussion

In the current study, we show that prolonged exposure of rat cortical neuronal cultures to ethanol enhanced NMDA stimulation of NOS activity. In contrast, chronic ethanol did not alter either kainate- or AMPA-stimulated NOS activity; did not alter NOS activation by the calcium ionophore ionomycin; and in the presence of MK-801 to block endogenous glutamate activation of NMDA receptors, did not alter KCl-stimulated NOS activity. Furthermore, immunoblot analysis showed that chronic ethanol did not alter the neuronal NOS protein levels. Taken together, these observations indicate that the selective enhancement of NMDA stimulation of NOS activity probably reflects an adaptive response at the cellular level to the effects of prolonged ethanol inhibition of NMDA receptors that does not involve an increase in the level of NOS protein or the calcium sensitivity of NOS. Although we did not measure intracellular calcium in the current study, other investigators, using Fura-2 to monitor intracellular Ca^{2+} concentration in primary cortical neuronal cultures (7) and cerebellar granule cell cultures (6), have shown that chronic ethanol exposure enhances NMDA-stimulated increases in intracellular Ca^{2+} concentration. These studies also demonstrated that in contrast to NMDA, chronic ethanol did not alter KCl-stimulated increases in intracellular Ca^{2+} concentration. These observations are in agreement with the results of the current study showing that chronic ethanol enhanced NMDA but not AMPA-, kainate-, KCl-, or ionomycin-stimulated increases in intracellular Ca^{2+} concentration.

Recent behavioral studies suggest that NO is involved in alcohol dependence and withdrawal. Competitive inhibitors of NOS were shown to impair the development of rapid tolerance to loss of motor control and to decrease the severity of ethanol withdrawal, whereas NO donors produced the opposite effect in that they increase the severity of withdrawal signs and symptoms (10, 11, 13). Our observation that chronic ethanol can potentiate NMDA-stimulated NO formation may implicate hyperfunctioning of NO signaling in the expression of ethanol dependence and withdrawal. Alcohol dependence and tolerance are diagnostic criteria for alcoholism, and alcoholics often achieve blood levels of ethanol during severe intoxication comparable to those used in the current study (i.e., 100 mM). Although intoxication occurs at lower blood ethanol levels (e.g., 20 mM), some neurochemical responses observed at higher concentrations also occur at lower concentrations with longer exposure times (18). In any case, our finding would clearly be expected to occur in alcoholism and perhaps after extended periods of intoxication.

NO is an important biological mediator in a number of physiological and pathological processes and seems to play a particularly important role in actions linked to NMDA receptors. Studies of hippocampal long term potentiation have shown that NO is involved in the maintenance of long term potentiation by acting as a retrograde messenger to increase presynaptic neurotransmitter release after tetanic stimulation of postsynaptic NMDA receptors. Thus, disruption of

normal NO production, which could involve either a reduction in NO formation due to the inhibitory effect of acute ethanol or an increase in NO formation after chronic ethanol, may contribute to the amnesia and memory impairment often associated with ethanol abuse. NO is also a known cytotoxic agent that has been linked to NMDA receptor neurotoxicity (excitotoxicity). Chronic exposure of neuronal cultures to ethanol can sensitize neurons to excitotoxicity, and it follows that enhanced NMDA-stimulated NO formation may play a role in alcohol-related brain damage. Furthermore, NO is thought to play a role in other processes affected by chronic ethanol, including neuronal development, aggression, and libido. Thus, altered NO formation and NMDA receptor activity may be a common mediator of the effects of chronic ethanol on brain function. Long term (3 month) chronic ethanol exposure in rats has been reported to decrease the levels of neuronal NOS in the frontal-parietal cortex, hippocampus, and striatum but to increase neuronal NOS levels in the nucleus accumbens (19).

In vitro studies have shown that chronic ethanol can potentiate NMDA-stimulated Ca^{2+} flux (6, 7), NO formation (current study), and neurotoxicity (8, 20), whereas *in vivo* studies show that NMDA antagonists can decrease the occurrence and severity of the ethanol hyperexcitability/withdrawal syndrome (21–23). Although these observations indicate that prolonged attenuation of glutamatergic neurotransmission by ethanol results in a compensatory increase in NMDA receptor function, the exact mechanism by which this adaptive increase occurs is not clear. A common cellular mechanism for restoring normal receptor activity in response to prolonged inhibition is an increase in receptor density. Some studies have reported increases in NMDA receptor binding after chronic ethanol treatment (7, 24, 25), whereas others have not (26, 27). This observation seems to depend on such factors as the particular ligand and binding conditions, animal type, brain region examined, and method and length of ethanol administration. An alternative means of assessing NMDA receptor density is to determine the level of expression of NMDA receptor subunits. Expression of cloned NMDA receptor subunit proteins in oocytes indicates that the NR1 subunit is an obligatory component of native NMDA receptors. As is the case with receptor binding studies, studies examining the levels of NR1 mRNA and NR1 protein have also produced conflicting observations that seem to depend on such factors as the length, time, and method of chronic ethanol administration; particular animal species; and brain region examined (7, 28–30). In the current study, membranes isolated from control and chronic ethanol-exposed cultures did not differ in specific binding of the noncompetitive NMDA antagonist ^{125}I-MK-801 or in the level of expression of the NR1 subunit protein. Taken together, these observations suggest that the increase in NMDA receptor function (as demonstrated by sensitization to NMDA excitotoxicity and enhanced NMDA-stimulated NO formation) produced by our ethanol-exposure paradigm is not due to an obvious increase in the density of NMDA receptors.

Transfection studies in oocytes have shown that although NR1 is an obligatory subunit, expression with one or more of the NR2 subunits is necessary to form receptors that functionally resemble native NMDA receptors (31). Furthermore, coexpression of NR1 with different NR2 subunits yields hetero-oligomeric receptors with different physiological and pharmacological properties. This has led to the suggestion that NR2 subunits confer functional heterogeneity on NMDA receptors, including sensitivity to ethanol (32). Immunohistochemical mapping studies have shown that the rat cerebral cortex contains NR2A and NR2B subunits and little or no NR2C or NR2D subunits (7, 33), and most NMDA receptors in the cerebral cortex are thought to be composed of one or more NR1 subunits expressed with one or more NR2A and/or NR2B subunits. A differential change in NR2 subunit gene expression in response to chronic ethanol exposure could yield hetero-oligomeric NMDA receptors that show increased function without an increase in receptor density. However, immunoblot analysis of NR2A and NR2B subunit polypeptides using subunit selective antibodies in the present study showed that chronic ethanol did not produce a differential change in expression of the NR2A or NR2B subunits. There also was no change in the overall expression of the NR2A and NR2B subunits, further indicating a lack of change in NMDA receptor density. The expression of other NR2 subunits was not examined because NR2A and NR2B are the predominant NR2 subunits in the cerebral cortex and primary cortical cultures. In addition to NR2 subunits, a number of other factors may contribute to the functional heterogeneity of NMDA receptors. For example, different splice variants of NR1 show differences in sensitivity to ethanol when expressed as homomeric receptors in oocytes (34). It is also possible that other nontraditional types of NMDA receptors, such as the glutamate binding proteins previously described (35), may further contribute to heterogeneity of NMDA receptor-mediated responses. Post-translational modifications are also likely to play a roll in regulating NMDA receptor activity. Studies have shown that both tyrosine and serine/threonine kinases and phosphatases modulate NMDA receptors activity (36–40), and phosphorylation/dephosphorylation of the NMDA receptor complex or associated proteins is probably an important means of regulating long and short term changes in glutamatergic transmission, including changes in response to chronic ethanol exposure. Finally, another post-translational modification that could influence NMDA receptor activity may involve NO itself. NO (or an NO-related species) has been reported to reduce NMDA receptor activity by *S*-nitrosylation at a critical site or sites on subunit proteins. Although speculative, loss or desensitization of NO feedback inhibition during chronic ethanol could result in supersensitization of NMDA responses.

Although the current study does not address the site of action of ethanol on the NMDA receptor, we previously found that ethanol inhibition of NMDA-stimulated NO formation is dependent on the presence of Mg^{2+} in a concentration-dependent manner (2). In the current study, chronic ethanol did not change the Mg^{2+} sensitivity of NMDA-stimulated NOS activity. Interestingly, the inhibitory effect of ethanol on NMDA-stimulated NOS activity decreased from ~60% to ~28% after chronic ethanol exposure. Thus, prolonged inhibition of NMDA receptors by ethanol may produce compensatory increases in both NMDA receptor function and a reduction in sensitivity to the inhibitory effect of ethanol on NMDA receptors that couple to NO formation. This reduced sensitivity could be due to a number of factors, including undetected changes in subunit stoichiometry of the hetero-oligomeric receptor complex, altered phosphorylation state of

the receptors, or changes in modulation by receptor-associated proteins.

In summary, we demonstrated that prolonged exposure of cortical cultures to ethanol potentiates subsequent stimulation of NO formation in the absence of ethanol. This enhancement was observed only with NO formation coupled to NMDA receptors. Hyperfunctioning of NMDA receptor-dependent NO signaling after chronic ethanol exposure may contribute to a variety of alcohol-related effects on the brain, including memory impairment and neurotoxicity. The increase in NMDA receptor function that we observed in response to chronic ethanol did not seem to result from a simple up-regulation of NMDA receptor density or to an obvious change in NR2 subunit composition of the hetero-oligomeric receptor complex; it may reflect more subtle changes in long term NMDA receptor modulation.

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