

Evidence for a Causative Role of N-Methyl-D-aspartate Receptors in an *In Vitro* Model of Alcohol Withdrawal Hyperexcitability¹

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Accepted for publication May 22, 1998 This paper is available online at <http://www.jpet.org>

ABSTRACT

Synaptic mechanisms underlying hyperexcitability due to withdrawal from chronic ethanol exposure were investigated in a hippocampal explant model system using electrophysiological techniques. Whole-cell voltage clamp recordings from CA1 pyramidal cells demonstrated that acute ethanol exposure inhibited N-methyl-D-aspartate receptor (NMDAR)-mediated excitatory postsynaptic currents by over 40%. Chronic ethanol exposure for 6 to 11 days at 35 or 75 mM induced no differences from control explants in the fast component of the population synaptic response (non-NMDAR-mediated). Prolonged field potential recordings (to 10 hr) were used to monitor the withdrawal process *in vitro*. Ethanol-exposed explants from both 35 and 75 mM groups displayed an increase (60% and 89%, respectively) in the NMDAR-mediated component of synaptic transmission on withdrawal from chronic exposure. Prolonged tonic-clonic electrographic seizure activity was consistently

observed after ethanol withdrawal only after the increase in NMDAR function. This hyperexcitability was inhibited by the NMDAR antagonist D-2-amino-5-phosphonovaleric acid and returned once the NMDAR component was reestablished after antagonist washout. *In situ* hybridization studies suggest that expression of NR2B subunit mRNA may be enhanced in explants after chronic ethanol exposure. No lasting differences were observed in the NMDAR component after acute *in vitro* ethanol exposure and withdrawal. These data suggest that the occurrence of ethanol withdrawal hyperexcitability in this system may be directly dependent on alterations in NMDAR function after chronic exposure. Since this region and others that contain ethanol sensitive NMDARs may serve as epileptic foci, long term alterations in NMDAR function may be expected to generate paroxysmal depolarizing shifts underlying ictal events after withdrawal from ethanol exposure.

Abstinence after chronic exposure to ethanol often leads to a physical withdrawal syndrome including symptoms in humans such as tremor, agitation, delirium and in severe cases, convulsions and brain damage (Victor and Brausch, 1967; Tabakoff and Rothstein, 1983). NMDARs are a subclass of excitatory neurotransmitter receptors that play important physiological roles in vertebrate nervous systems (McBain and Mayer, 1994). Since studies have demonstrated that intoxicating concentrations of ethanol inhibit the NMDAR (Hoffman *et al.*, 1989; Lovinger *et al.*, 1989), it is conceivable that a substantial component of alcohol withdrawal hyperexcitability may be related

to an adaptive upregulation of NMDAR function due to chronic exposure. Evidence supporting this hypothesis has accumulated over the past few years. Grant *et al.* (1990) demonstrated an increase in the number of binding sites for the NMDAR channel blocker, MK-801, in the hippocampus after chronic ethanol exposure. These investigators and others (Morrisett *et al.*, 1990) showed that MK-801 protected against withdrawal hyperexcitability in rodents. This effect has also been demonstrated using a competitive NMDAR antagonist (CGP39551) *in vivo* (Liljequist, 1991) and in hippocampal slices taken from rats chronically exposed to ethanol (Ripley and Little, 1995). Biochemical studies have demonstrated an increased sensitivity to NMDA in cultured neurons after chronic ethanol exposure (Iorio *et al.*, 1992; Chandler *et al.*, 1993; Blevins *et al.*, 1995). Recently, electrophysiological evidence for a chronic ethanol-induced enhancement of NMDAR-mediated synaptic responses was provided by Whittington *et al.* (1995) in hippocampus. Taken together these studies suggest a role for the NMDAR in

Received for publication February 12, 1998.

¹ This work was supported by Grant R299230 from the National Institute of Alcohol Abuse and Alcoholism to R.A.M.

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CE, chronic ethanol-treated; D-APV, D-2-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; EGS, electrographic seizure; EPSC, excitatory postsynaptic current; GABA, gamma-aminobutyric acid; NMDAR, N-methyl-D-aspartate receptor; PBS, phosphate-buffered saline; PS, population spike; PSP, postsynaptic potential; SSC, saline-sodium citrate.

the alterations in neural function due to chronic ethanol exposure.

In this study, we test the hypothesis that NMDARs play a critical role in the expression of ethanol withdrawal hyperexcitability. Specifically, we have analyzed the temporal relationship between the alteration in NMDAR function and the occurrence of neuronal hyperexcitability due to removal from chronic ethanol exposure. We propose that several criteria must be satisfied to provide strong support for this hypothesis. First, direct evidence must be provided of enhanced NMDAR function on ethanol withdrawal in comparison with nonethanol-exposed or nonwithdrawn tissue. Second, the tissue must exhibit evidence of withdrawal hyperexcitability. Third, a temporal relationship should exist such that the enhanced NMDAR function should precede (or occur in parallel with) the withdrawal hyperexcitability. Fourth, the withdrawal hyperexcitability should be prevented by antagonists of NMDAR function. Finally, the altered NMDAR function and withdrawal hyperexcitability should be dependent on chronic exposure and not observed with acute exposure.

Several experimental systems have been utilized to study the cellular and molecular mechanisms mediating ethanol withdrawal hyperexcitability (Trevisan *et al.*, 1994; Blevins *et al.*, 1995; Whittington *et al.*, 1995; Hu *et al.*, 1996). While the existing model systems have provided important evidence regarding these mechanisms, an *in vitro* system that enables the observation of synaptic responses during seizure expression is preferable. Brain slice culture preparations may provide such a system for the study of chronic ethanol effects on neural function. Recently, a technique has been developed in which brain slices are maintained in culture on a porous membrane at an air-medium interface (Stoppini *et al.*, 1991). The general morphology and synaptic connections of neurons in organotypic hippocampal slices cultured using this interface technique largely resemble that seen in native tissue (Buchs *et al.*, 1993), and analyses of several molecular markers suggest many similarities to mature brain (Bahr, 1995). The explants exhibit excitatory and inhibitory synaptic responses and several forms of synaptic plasticity including NMDAR-dependent long-term potentiation (Stoppini *et al.*, 1991; Muller *et al.*, 1993). Of particular relevance for the study of postwithdrawal hyperexcitability, hippocampal explants are also capable of generating epileptiform burst discharges and tonic-clonic seizure events under certain conditions (McBain *et al.*, 1989).

We used electrophysiological techniques to assess the effects of acute and chronic ethanol exposure and withdrawal on neural excitability associated with NMDAR function in hippocampal explant cultures. These techniques included whole-cell voltage clamp as well as field potential recordings to maintain long-term recordings during the withdrawal period. Additionally, recent evidence suggests that the upregulation of NMDAR function after chronic ethanol exposure may involve the NR2B subunit of the heteromeric receptor complex (Trevisan *et al.*, 1994; Hu *et al.*, 1996). We thus also used *in situ* hybridization techniques to address whether alterations in NR2B subunit message occur in the explants after chronic ethanol exposure. Some of the data reported here have been presented previously in abstract form (Thomas *et al.*, 1995, 1996).

Methods

Slice preparation and culture. Slices used in this study were prepared from 9- to 11-day-old Sprague-Dawley rat pups of both sexes. The animals were anesthetized with halothane and decapitated, and the brain was rapidly removed and placed in ice-cold oxygenated ACSF for 3 to 4 min. The hippocampi were then removed bilaterally and 500 micron transverse sections were cut, transferred to a holding chamber containing ACSF bubbled with 95% O₂/5% CO₂ (carbogen) and maintained at 32° to 35°C. ACSF consisted of (in mM): NaCl, 120; NaHCO₃, 25; KCl, 3.3; NaH₂PO₄, 1.2; CaCl₂, 1.8; MgSO₄, 2.4; dextrose, 10.

Slices were cultured using a modification of methods described by Stoppini *et al.* (1991). After incubation in ACSF at 32° to 35°C for 1 to 2 hr, slices were transferred onto membrane inserts (Millicell-CM; Millipore Corp., Bedford, MA) in 6-well plates containing 1 ml of culture medium/well. The plates were placed in an incubator at least 15 min before tissue transfer to allow the pH and temperature of the medium to stabilize. The cultures were then maintained in the incubator at 37°C in a 5% CO₂-enriched atmosphere. The culture medium consisted of 75% minimum essential medium (GIBCO 12360; Gaithersburg, MD), 25% heat-inactivated horse serum (GIBCO 26050), glutamine (3 mM), glucose (5.5 mg/ml medium) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively; GIBCO 15140). Medium was replaced on the day after slice preparation and then on alternate days.

Prior to recording, slice cultures were transferred to 35-mm petri dishes mounted on the stage of an inverted microscope. The slices were submerged in and continuously perfused with, carbogen-saturated ACSF at a flow rate of ~1.5 ml/min (Rainin Rabbit-Plus peristaltic pump, Woburn, MA). The recording ACSF was the same as that used for tissue preparation except that the concentrations of CaCl₂ and MgSO₄ were 2.0 mM and 0.9 mM, respectively (except where noted). The tissue was perfused in the acute recording solution for 1 hr before beginning recordings to insure washout of the culture medium. Drugs were dissolved in ACSF and applied to the tissue by switching solution reservoirs. All drugs were obtained from Sigma (St. Louis, MO) except CGS-19755, which was obtained from Nova Pharmaceuticals (Baltimore, MD), and ethanol (95%), from University of Nebraska Medical Center Hospital Supply.

Patch clamp recording of NMDAR-mediated synaptic currents. Tight-seal whole-cell patch recordings were made at room temperature from CA1 pyramidal neurons using techniques previously described (Morrisett and Swartzwelder, 1993). Recording electrodes were made from thin-walled borosilicate glass (TW150F-4, WPI, Sarasota, FL, 1.2–2.2 MΩ) and filled with (in mM): CsMeSO₃, 135; NaCl, 8; EGTA, 0.5; HEPES, 10; MgCl₂, 2; Tris-ATP, 2; Tris-GTP, 0.3; 260–270 mOsm, pH 7.2 with CsOH. Access resistance was partially compensated after cell break-in and monitored throughout the experiment. Recordings where access resistance increased more than 10% during the course of the experiment were not included in data analyses. EPSCs were evoked by stimulation of Schaffer collateral fibers in the stratum radiatum layer of area CA3 via monopolar tungsten electrodes. Constant-current pulses (100 μsec duration, 9–34 μA amplitude) were applied through a stimulus isolation unit driven by a digital stimulator (Master-8, A.M.P.I., Israel). Recordings were made using a Warner PC-501A amplifier (Warner Instrument Corp., Hamden, CT), filtered at 1 kHz and digitized at 10–20 kHz using a Lab Master DMA interface (Scientific Solutions, Solon, OH). Data acquisition and device control were implemented using AxoBASIC software (Axon Instruments Inc., Foster City, CA).

The NMDAR-mediated component of synaptic current was recorded after pharmacological elimination of other major synaptic components. The fast, non-NMDA EPSC was blocked by bath application of 10 μM DNQX. The fast IPSC was eliminated by holding the membrane potential near E_{Cl} (approximately -50 mV). The slow, GABA_B receptor-mediated K⁺ current was eliminated by including Cs⁺ in the patch pipette solution. Finally, activation of NMDARs was

facilitated by lowering the extracellular Mg^{2+} concentration to 0.1 mM during patch clamp recording.

Chronic ethanol exposure. Slice explants were cultured for 6 to 7 days before exposure to ethanol, and explants prepared from the same animals were then randomly distributed between the control and ethanol groups. Slices were exposed to either 35 mM (7–11 days) or 75 mM (6 days) ethanol. Ethanol was added to the preequilibrated medium at the desired concentration and the culture plates were placed in a sealed, humidified plastic container to prevent evaporation. No changes in pH were noted using this exposure paradigm and no differences in field potentials were noted between explants cultured in the containers and normal explants (data not shown). Ethanol was added to the water in the chamber floor at a slightly elevated concentration (empirically determined to be 90 mM to give 75 mM and 42 mM to give 35 mM, Sigma kit 333-A) to prevent evaporation loss on establishing equilibrium. Control slices for the two ethanol groups were maintained in standard culture medium in separate sealed, humidified containers. Media ethanol concentrations were monitored regularly using a standard diagnostic kit (Sigma 333-A).

Extracellular recording. Experiments on control and ethanol-exposed slices were performed on alternate days. The extracellular recordings used to monitor synaptic potentials before and during ethanol withdrawal were performed in normal recording ACSF, that is, with no receptor antagonists, 0.9 mM $MgSO_4$ and 2.0 mM $CaCl_2$. Population field potentials were recorded at $32 \pm 1^\circ C$ from the CA1 pyramidal cell layer with glass microelectrodes filled with 150 mM NaCl (1–3 M Ω). Synaptic responses were evoked by stimulation of Schaffer collateral fibers and the responses were amplified using a DC-coupled amplifier, with data acquisition and analysis implemented as described above for patch clamp recordings.

Plots of the population synaptic potential (PSP) amplitude vs. stimulus amplitude (input/output curves) were obtained after an initial 1-hr period of equilibration in ACSF \pm ethanol. Averaged responses ($n = 5$) were then collected hourly at a stimulus intensity that evoked a near maximal peak PSP. Recordings were made for 2 hr before withdrawal of ethanol and for up to 7 hr after withdrawal in ethanol-exposed slices. Input/output curves were obtained near the end of the recording period, and in some experiments were obtained hourly during the entire experiment. Spontaneous and evoked electrographic seizure events were recorded using a digital tape recorder (Sony DAT Model 75ES).

In situ hybridization. Standard techniques were used to detect the presence of mRNA for the NR1, NR2A and NR2B receptor subunits (see Monyer *et al.*, 1992, for oligonucleotide sequences). Antisense oligonucleotide probes were 3' end-labeled with α - ^{35}S -dATP using terminal deoxynucleotidyl transferase (New England Nuclear, Boston, MA) and unincorporated nucleotide was removed by Nensorb chromatography (New England Nuclear). After a brief rinse in chilled phosphate-buffered saline (PBS, pH 7.3) the tissue slices were fixed on the membrane insert for 5 min in 4% paraformaldehyde in PBS, rinsed with chilled PBS, removed from the inserts, freeze-mounted to slides (Superfrost Plus, Fisher Scientific, Pittsburg, PA) and stored frozen overnight. The tissue was then immersed in chilled 70% ethanol for 3 min and stored in 95% ethanol at $-20^\circ C$. ^{35}S -Labeled oligonucleotide probe was dissolved in hybridization buffer (2000 cpm/ μ l; New England Nuclear) containing 0.2 M dithiothreitol and applied to the tissue slices, which were then sealed in special slide chambers (Probe Clip, RPI Corp., Mount Prospect, IL). The tissue was incubated overnight at $42^\circ C$ (NR1 and NR2A) or $48^\circ C$ (NR2B). After a high-stringency rinse (1 hr at $60^\circ C$ for NR1 and NR2A, 20 min at $65^\circ C$ for NR2B) in $1\times$ SSC buffer (0.015 M sodium citrate, 0.15 M NaCl), the tissue was incubated at room temperature for 5 min each in $1\times$ SSC, $0.1\times$ SSC, 70% ethanol and 95% ethanol and then air-dried. Slides were then applied to film (β max; Amersham, Arlington Heights, IL) and developed for 2 days (NR1), 4 days (NR2A) or 4 to 6 days (NR2B).

Data analysis. Measures were expressed as mean \pm S.E.M. for all experiments. Drug effects were evaluated using Student's *t* test, and differences between chronic ethanol-treated and control groups for input-output curves and time course experiments were evaluated using ANOVA followed by Bonferroni corrected comparisons between the intervals indicated for each experiment. Differences were considered significant at a confidence interval of $P < .05$.

A quantitative analysis of NR2B subunit-specific oligonucleotide probe density was performed on explant tissue subjected to *in situ* hybridization after chronic ethanol exposure using image analysis software (MCID, Imaging Research, St. Catherines, Ontario, Canada). Probe density per slice was estimated by averaging values from 10 circular regions in the cell body layers of area CA1, CA3 and dentate gyrus, calibrated to a density standard curve to give relative probe density. Mean values for control and chronic ethanol-treated slices were compared using Student's *t* test.

Results

Acute effects of ethanol on NMDAR-mediated synaptic responses. NMDAR-mediated EPSCs were recorded from 20 CA1 pyramidal cells in hippocampal explants that were cultured for 8 to 33 days. NMDA EPSCs recorded in the explants showed kinetic and pharmacological properties characteristic of currents mediated by this subtype of glutamate receptor. Figure 1 shows the inward current evoked in a CA1 pyramidal cell by stimulation of Schaffer collaterals in an explant slice after 8 days *in vitro*. The stimulus intensity for all patch clamp recordings was adjusted to evoke the maximal inward current obtainable without apparent loss of voltage clamp (activation of voltage-dependent conductances). The mean peak amplitude for the evoked EPSCs was 172 ± 13 pA and ranged from 65 to 265 pA ($n = 20$). The evoked currents exhibited the relatively slow rise and decay times characteristic of NMDAR-mediated synaptic currents. The mean rise time for the NMDA EPSCs was 28 ± 1.4 msec with a range from 18 to 41 msec ($n = 20$), while the current decay was fitted to a single exponential curve with a mean time constant of 140 ± 22 msec ($r = 0.96 \pm 0.01$, $n = 7$). The evoked currents were reversibly blocked by bath application

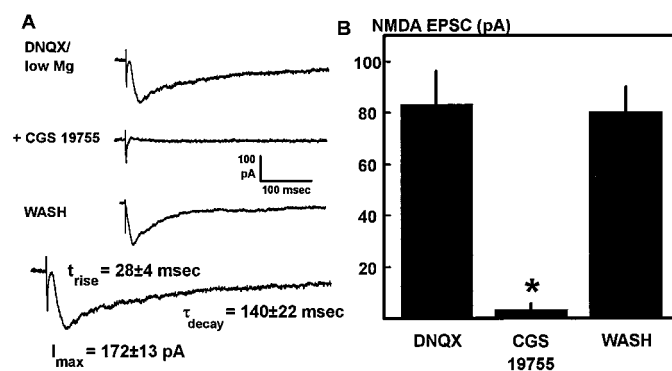


Fig. 1. NMDAR-mediated EPSCs show characteristic time course and pharmacology in hippocampal explants. A, Evoked EPSCs recorded from a CA1 pyramidal cell in an explant after 8 days in culture (stimulus amplitude = $20 \mu A$; holding potential = -52 mV; all traces are average of 5 responses). Traces are shown for the current evoked in $10 \mu M$ DNQX and 0.1 mM Mg^{2+} , after perfusion with $2 \mu M$ CGS-19755, and after drug washout. Trace at bottom shows control waveform with mean values for key parameters. B, Mean amplitude of NMDA EPSCs ($n = 4$ cells) in control solution, during perfusion with $2 \mu M$ CGS-19755 and after washout of drug. Inhibition by CGS-19755 was significant (Student's *t* test, $P < .05$), and mean amplitude after drug washout was not different than control value. The amplitude of the responses was measured 100 msec after the shock artifact.

of 2 μM CGS-19755 ($97 \pm 3\%$ inhibition, $n = 4$ cells), a specific NMDAR competitive antagonist (Lehmann *et al.*, 1988), confirming that they were mediated by activation of NMDA receptors (fig. 1).

Bath application of ethanol reversibly inhibited NMDA EPSCs recorded from CA1 pyramidal cells in the explants (fig. 2). In the recording shown, application of 75 mM ethanol reduced the EPSC by 60% relative to the control response measured at a latency of 100 msec from the stimulus artifact. Exposure to 75 mM ethanol reduced the amplitude of NMDA EPSCs on average by $42 \pm 6\%$, with a range of inhibition from 18 to 60% ($n = 6$ cells from explants 9–28 days *in vitro*; the level of inhibition was not significantly correlated with explant age). This inhibition was reversible after washout of ethanol, returning to $95 \pm 8\%$ of control amplitude ($n = 5$ cells). Thus, the NMDAR-mediated component of synaptic transmission in the hippocampal explants is inhibited by ethanol to a similar extent as previously demonstrated for native hippocampal tissue (Lovinger *et al.*, 1989; Morrisett and Swartzwelder, 1993).

Since we eliminated GABA_A-mediated currents by clamping near E_{Cl} , it is conceivable that an ethanol-induced enhancement of GABA_A channel activity could lead to an apparent decrease in NMDAR current via a membrane shunting effect. This seems unlikely, however, because the resulting change in membrane time constant would likely alter the kinetics of the EPSC decay, and scaling of EPSC waveforms evoked in the presence of ethanol to control waveforms (fig. 2, bottom) indicate that no change in decay kinetics occurs.

Chronic effects of ethanol on population field recordings. Population recordings were used to assess the effects of chronic ethanol exposure on excitatory synaptic function in the explants. A highly reproducible population waveform was recorded with an electrode placed in the cell body layer of area CA1 after stimulation of fibers in stratum radiatum of area CA3 (presumed Schaffer collaterals). The waveform evoked consisted of a slow positive potential

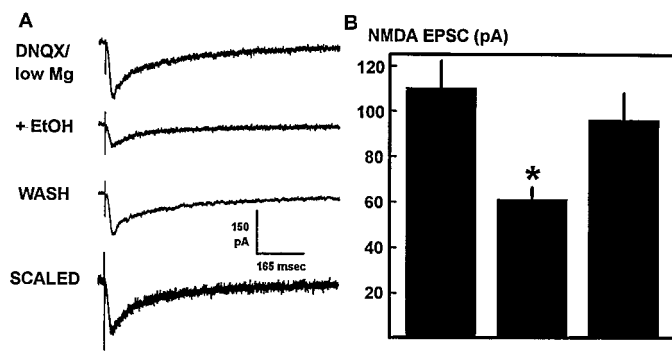


Fig. 2. Ethanol inhibits NMDAR-mediated EPSCs in hippocampal explants. A, Evoked NMDAR-mediated EPSCs recorded from a CA1 pyramidal cell in an explant after 14 days in culture (stimulus amplitude = 12 μA ; holding potential = -50 mV; all traces are average of 5 responses). Ethanol was applied for 15 min, and effects were seen within 3 to 5 min. Wash responses were obtained 15 to 30 min after switching back to control solution. Trace at bottom shows control response overlapped with ethanol response scaled to the same peak amplitude. B, Mean EPSC amplitude ($n = 6$ cells) in control solution, during exposure to 75 mM ethanol and after washout of ethanol. Inhibition by 75 mM ethanol was significant (Student's *t* test, $P < .01$), and mean amplitude after drug washout was not different than control value. The amplitude of the responses was measured 100 msec after the shock artifact.

which, at higher stimulus amplitudes, was superimposed a sharp, negative-going spike at a variable latency from the shock artifact (fig. 3A). These potentials correspond to the PSP and PS, respectively, recorded with this electrode configuration in acute hippocampal slices. In this study we focused on the field PSP as a general measure of synaptic function.

As seen in acute slices, increases in stimulation intensity resulted in a graded increase in the peak amplitude of the PSP from a threshold value until a maximal PSP was evoked (fig. 3A). Figure 3B shows a plot of the PSP peak amplitude *vs.* stimulus current amplitude for the waveforms shown in figure 3A. Note that the amplitude of the PSP rises steeply as a function of stimulus intensity; the response grades from threshold to maximal amplitude over a 5 μA range of stimulus amplitude. Further increases in stimulus amplitude increased the PS amplitude and tended to lead to a decrease in the observed peak of the PSP. At higher stimulus amplitudes, a slowly decaying component of the PSP was observed (fig. 3A, bottom waveform). This component appeared at stimulus amplitudes that evoked near-maximal peak PSP responses. Test responses, recorded hourly during the recording period, were evoked at a stimulus amplitude that produced a near-maximal peak PSP and a measurable slow component.

The relationship between PSP peak amplitude and stimulus intensity was not changed after chronic ethanol exposure, neither before nor after the withdrawal period (fig. 4). Figure 4A shows cumulative input/output plots obtained before and after the withdrawal period comparing data from 6 control slices and 8 slices exposed to 75 mM ethanol for 6 days. No difference was observed between chronic ethanol-treated (CE) and control groups for the threshold stimulus intensity (8.3 ± 1.1 μA for ethanol *vs.* 8.3 ± 1.2 μA for controls, fig. 4B). The maximal PSP peak amplitude (measured at twice the threshold stimulus) was 4.0 ± 1.0 mV for CE slices before the withdrawal period compared to 4.0 ± 0.7 mV for control slices. No significant difference was seen between input/output curves obtained from these same sets of slices 7 hr into the withdrawal period. Thus, the threshold stimulus intensity was 8.7 ± 1.5 μA for CE slices *vs.* 8.0 ± 1.1 μA for control slices (fig. 4B) and the maximal PSP peak amplitude was

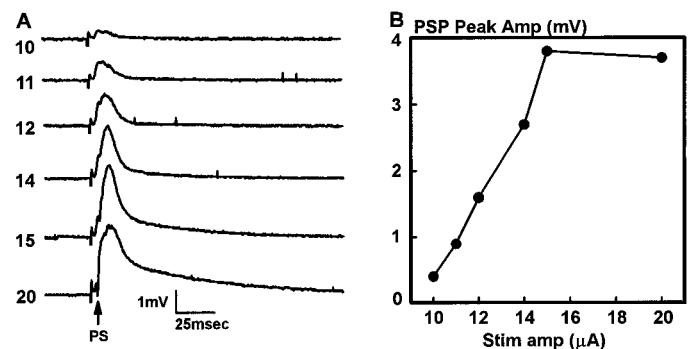


Fig. 3. Population PSP recordings in explants show steep input/output relationship. A, Representative population synaptic potentials (PSPs) evoked in a hippocampal explant at several stimulus intensities (shown to the left in μA). Note that at the two highest stimulus amplitudes, a slowly relaxing component appears in the PSP waveform (especially prominent at 20 μA). The PS indicated by the arrow is just noticeable at 20 μA . B, PSP peak amplitude plotted *vs.* stimulus amplitude for the evoked responses shown in A.

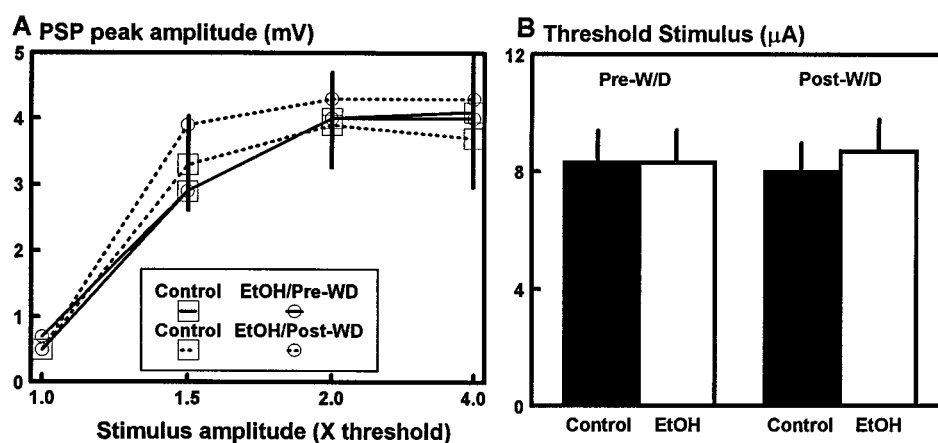


Fig. 4. PSP input/output curves are unchanged after chronic ethanol exposure and withdrawal. A, Plot of PSP peak amplitude *vs.* stimulus amplitude for control and 75 mM chronic ethanol (CE) explant slices before ethanol withdrawal (solid lines) and at the end of the recording period (dotted lines). The stimulus amplitude was normalized to the threshold intensity for each experiment for comparison between slices. Error bars (\pm S.E.M.) are shown only for the postwithdrawal control slices for clarity and were similar for the other groups. No significant differences in the curves were observed, either before or after withdrawal, between 75 mM CE and control groups (ANOVA, comparing 1 \times , 1.5 \times , and 2 \times threshold values for 75 mM CE, $n = 8$ and control groups, $n = 6$). B, Mean stimulus amplitude for evoking a measurable PSP for 75 mM CE and control groups before and after withdrawal.

4.3 \pm 0.8 mV for the CE group compared to 3.9 \pm 0.8 mV for controls.

Differences in the slow synaptic component were apparent in chronic ethanol-treated explants. Figure 5 shows evoked waveforms recorded from an explant slice after chronic exposure to 35 mM ethanol compared to corresponding waveforms recorded from a control explant. Maximal evoked PSPs are shown for three recording conditions (and the corresponding periods for the control slice): before withdrawal of ethanol from the recording media, several hours after the withdrawal of ethanol and after application of the competitive NMDAR antagonist, D-APV. A comparison of the prewithdrawal waveforms indicates that, while the peak amplitudes of the PSPs were similar, the slow component was substantially larger in the waveform from the CE explant (38% of peak for the ethanol slice *vs.* 10% of peak for the control slice). After the removal of ethanol from the recording solution, the slow component was further enhanced (to 92% of peak compared to 23% for the control slice at the corresponding time period),

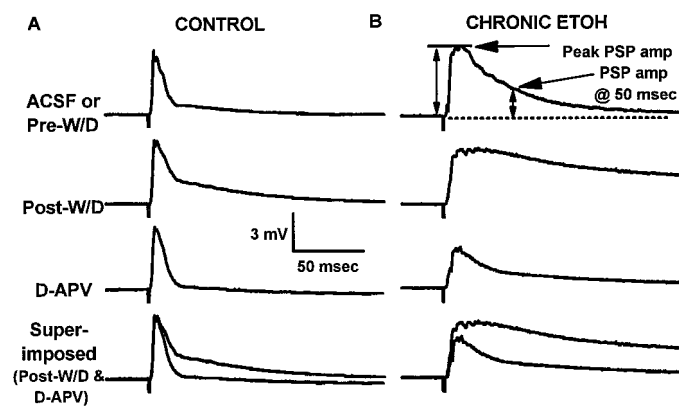


Fig. 5. Increase in NMDAR-dependent slow synaptic component after withdrawal from chronic ethanol exposure. Evoked PSPs recorded in a control explant (A) and from an explant exposed to 35 mM ethanol for 6 days (B). Traces are averages of 5 responses. Representative waveforms are shown for the prewithdrawal period, at 5 hr after withdrawal (Post-W/D), and after application of 25 μ M D-APV (bottom trace shows D-APV and postwithdrawal waveforms superimposed). At right, arrows indicate how the peak PSP amplitude and the PSP slow component amplitude (at 50 msec after the stimulus artifact) were measured.

greatly prolonging the duration of the PSP (fig. 5, post-W/D). This enhancement occurred without a significant change in the peak of the PSP over the duration of the recording period.

The slowly decaying component of the PSP was strongly inhibited by the application of D-APV in control and CE slices (fig. 5), indicating that this component is dependent on the activation of NMDA receptors. PSPs recorded in the presence of 25 μ M D-APV (*i.e.*, the non-NMDAR dependent component) were characterized by a rapidly decaying waveform that, in control explants from the 35 mM CE experiment, returned to near baseline by 50 msec from stimulus onset (fig. 5, control, D-APV). Note that for the 35 mM CE explant the slow component was not entirely eliminated in the presence of 25 μ M D-APV. Thus, for the control slice the PSP amplitude at 50 msec was reduced from 1.0 mV to 0 mV after application of 25 μ M D-APV, whereas in the 35 mM CE slice, D-APV reduced the PSP amplitude at 50 msec from 3.3 mV to 0.9 mV. For the 75 mM CE experiments, we used 50 μ M D-APV which virtually eliminated the slow component measured at 50 msec latency (see below). Thus, we used the amplitude of the PSP at 50 msec (fig. 5B) as an indirect measure of the NMDAR-dependent component because the waveform at this latency is relatively free from contamination by the non-NMDAR dependent component.

Figure 6 shows the time course of the slow, NMDAR-dependent synaptic component for all experiments in explants chronically exposed to 35 mM ethanol (fig. 6A) and 75 mM ethanol (fig. 6B). The graphs compare mean values at each time point for all experiments in the two CE groups with the values at corresponding time points for their respective control groups. Note that the slow component was larger in the 35 mM CE group before the removal of ethanol from the recording solution when compared with control explants (mean for 2-hr period was 35 \pm 8% of peak PSP for 35 mM ethanol, $n = 7$ and 17 \pm 6% of peak PSP for controls, $n = 4$, fig. 6A), although the difference was not significant. In contrast, no difference was observed before ethanol withdrawal in the 75 mM CE group relative to controls (2-hr mean was 27 \pm 4% of peak PSP for 75 mM ethanol, $n = 8$ and 24 \pm 4% of peak PSP for controls, $n = 5$, fig. 6B).

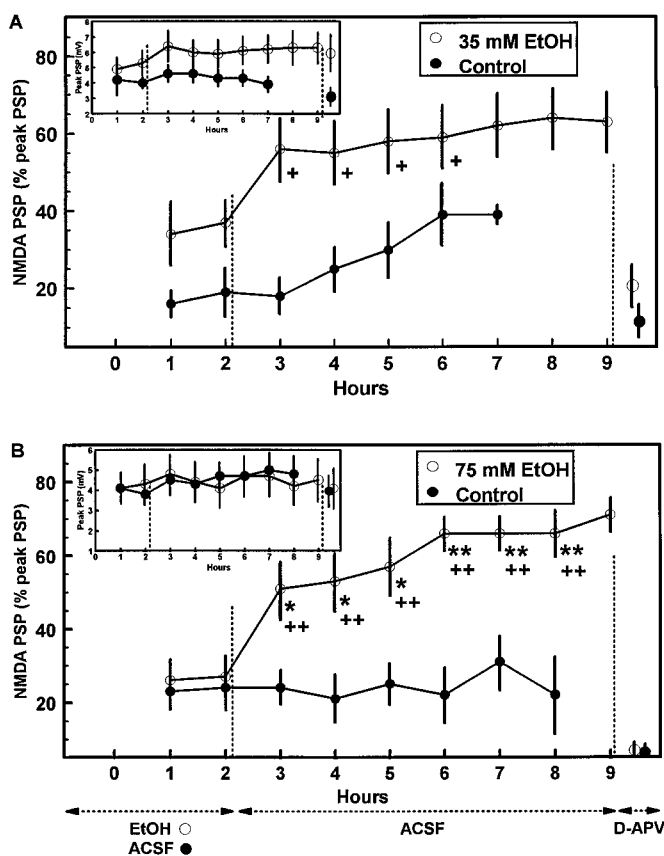


Fig. 6. Increase in PSP slow component after ethanol exposure occurs without a significant change in PSP peak amplitude. Plots of slow PSP component vs. time for all slices exposed to 35 mM (A) and 75 mM B, chronic ethanol. Responses were measured as shown in figure 5 and expressed as percent of peak PSP. Mean values \pm S.E.M. are shown for each time point (35 mM ethanol, $n = 7$; 35 mM control, $n = 4$; 75 mM ethanol, $n = 8$; 75 mM control, $n = 6$). Asterisks indicate time points where values for CE explants are different from corresponding control values, and plus signs indicate time points where postwithdrawal values are different from the 2-hr averaged prewithdrawal values (* = $P < .05$, ** = $P < .01$, + = $P < .01$, ++ = $P < .001$ or smaller; ANOVA followed by Bonferroni corrected comparisons at each time point). Final points show mean slow component in the presence of D-APV (25 μ M for 35 mM CE experiment, 50 μ M for 75 mM CE experiment). All measures in D-APV were significantly reduced compared with final predrug values (35 mM CE, $P < .001$, $n = 7$; 35 mM controls, $P < .05$, $n = 3$; 75 mM CE, $P < 1E-6$, $n = 8$; 75 mM controls, $P < .01$, $n = 5$; Student's t test). Insets show plots of PSP peak amplitude for the same experiments. Mean values \pm S.E.M. are shown for each time point. Values for the peak PSP were unchanged across time for both 35 mM (A) and 75 mM B, experiments, and values for CE explants were not different from corresponding control values (ANOVA). Final points in all plots show mean PSP peak amplitude in the presence of D-APV for both CE and control explants. Measures in D-APV were not significantly different compared with final predrug values for any group (Student's t test).

For both groups, a marked and significant increase in the slow component was observed within 1 hr from removal of ethanol from the perfusate. This increase occurred without a significant change in the peak of the PSP. In the 35 mM group, the NMDAR-dependent component increased on average from $35 \pm 8\%$ of peak PSP before withdrawal to $56 \pm 12\%$ of peak PSP 1 hr after withdrawal ($n = 7$), an increase of 60%. In the 75 mM group a larger relative increase of 89% was observed, from $27 \pm 4\%$ to $51 \pm 7\%$ of peak PSP ($n = 8$). Comparing hour by hour, the difference in the slow component between the 75 mM CE group and controls was significant during the entire withdrawal period (reaching $P < .01$

by 4 hr after withdrawal), where no significant change in the slow component was observed in control slices (fig. 6B, $n = 5$). Control slices interleaved with the 35 mM CE group did display a slow, modest increase in the slow component on average during the course of the experiment (fig. 6A, $n = 4$). This experiment was performed first, before the 75 mM experiment, and because no increase in the slow component was observed in the 75 mM controls, this slow increase may represent an artifact of experimental procedure. The slow component was significantly larger during the entire withdrawal period relative to the prewithdrawal period in the 35 mM CE explants; however, there was no statistically significant difference in the slow component between the CE and control groups during the withdrawal period.

The final points for each plot in figure 6 show the mean amplitude of the PSP slow component in the presence of D-APV (25–50 μ M). Compared to values measured just before drug exposure, 25 μ M D-APV significantly reduced the slow component from $63 \pm 9\%$ to $21 \pm 5\%$ of peak PSP in the 35 mM CE group ($n = 7$) and from $39 \pm 3\%$ to $7 \pm 4\%$ of peak PSP in the control group ($n = 3$, fig. 6A). Relative to predrug values, 50 μ M D-APV significantly reduced the slow component from $71 \pm 4\%$ to $4 \pm 2\%$ of peak PSP in the 75 mM CE group ($n = 8$) and from $22 \pm 11\%$ to $3 \pm 2\%$ of peak PSP in the control group ($n = 5$, fig. 6B).

The increase in the slow NMDAR-dependent PSP component that was observed after chronic ethanol exposure occurred without a significant change in the peak of the PSP. The insets in figure 6 show the time course of the peak PSP amplitude for all slices in the two ethanol groups and their corresponding controls. No significant change in peak PSP amplitude occurred in either experimental group at any time point. The final points for these plots show the mean peak PSP amplitude of the PSP in the presence of D-APV (25–50 μ M). These values were not significantly different from the mean peak PSP amplitude before drug application (5.8 ± 1.2 vs. 6.3 ± 1.0 mV predrug for 35 mM CE, $n = 7$ and 3.0 ± 0.6 vs. 3.9 ± 0.5 mV predrug for controls, $n = 3$; 4.1 ± 1.0 vs. 4.5 ± 1.1 mV predrug for 75 mM CE, $n = 8$ and 3.9 ± 0.8 vs. 4.8 ± 0.9 mV predrug for controls, $n = 5$) further demonstrating that the peak PSP can be used as a measure of the non-NMDAR mediated synaptic component.

Acute ethanol exposure and withdrawal. The enhancement of the slow synaptic component after withdrawal from chronic ethanol exposure could conceivably be due, at least in part, to a rebound of NMDAR function from the acute inhibitory effects of ethanol. To test for this possibility, we studied the effects of acute ethanol exposure and withdrawal on the slow synaptic component in recordings from ethanol-naive explants. Figure 7 shows the results of these experiments. Evoked PSPs were recorded before, during and for 3 hr after a 1-hr exposure to 75 mM ethanol. In these experiments, a stimulus intensity was used that produced a large slow component in the field PSP so that an acute inhibitory effect of ethanol could be readily observed. Application of ethanol to the bathing solution significantly decreased the slow PSP component to $70 \pm 10\%$ of the control value within 15 min ($n = 4$ slices). After washout of ethanol, the slow component returned toward control levels within 15 min, with a transient enhancement observed in 3 of 4 slices (mean of $126 \pm 12\%$ of control for all 4 slices at 1 hr after washout);

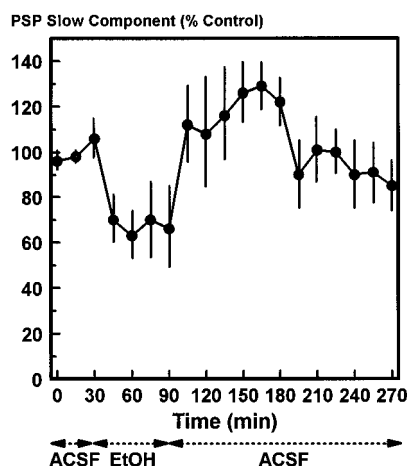


Fig. 7. Acute ethanol exposure does not result in lasting enhancement of NMDAR-dependent PSP component in ethanol-naive explants. Plot shows the slow PSP component before, during and for 3 hr after exposure to 75 mM ethanol for 1 hr. Explants ($n = 4$) were treated as described for previous control experiments, including placement in a humidified chamber for 6 days. Values are expressed as a percentage of the mean value calculated over the 3 intervals before ethanol exposure. The mean value for the 1-hr interval in ethanol was significantly reduced compared to the mean for the control period ($P < .05$), whereas mean values for each 1-hr interval after ethanol removal were not significantly different from the control period (ANOVA followed by Bonferroni corrected comparisons for each hour interval).

however, the response returned to control levels by 2 hr after washout.

Ethanol withdrawal and ictal events in explants. Explant slices that were exposed to chronic ethanol displayed a marked hyperexcitability during the period after withdrawal from ethanol. This electrical hyperactivity was manifested as spontaneous epileptiform burst discharges, large abrupt depolarizing shifts and long-lasting phasic or phasic/tonic electrographic seizure (EGS) events. The long-lasting EGSs often followed single electrical shocks applied to evoke PSPs but they occasionally occurred spontaneously, after large amplitude burst discharges. Figure 8 shows an EGS induced after a single stimulus in an explant slice after withdrawal from chronic exposure to 35 mM ethanol. This event lasted approximately 90 sec and showed a morphology that was typical of the phasic/tonic type of ictal events seen during withdrawal. After the initial evoked response (from less than a second to several seconds), ictal events typically were initiated by spontaneous population spikes (fig. 8a) which increased in frequency, producing a slow depolarization envelope (fig. 8b). This was followed by high-amplitude, phasic bursts that often led to a period of tonic firing (fig. 8c). Seizure events that progressed from phasic to tonic bursting eventually returned to a phasic bursting phase. Finally, the hyperpolarizations that followed phasic bursts (fig. 8d) gradually increased in amplitude, terminating the spontaneous bursting activity (fig. 8e) and resulting in a quiescent period where excitability (evoked and spontaneous) was greatly reduced. During this quiescent period, spontaneous epileptiform burst discharges and depolarizing shifts were much less frequent or entirely absent, and evoked PSPs were greatly reduced and required 30 min or more to return to their previous amplitude.

The ictal events were all-or-none in nature, and their duration tended to be similar in a particular explant. Thus, once initiated they exhibited a full phasic/tonic discharge se-

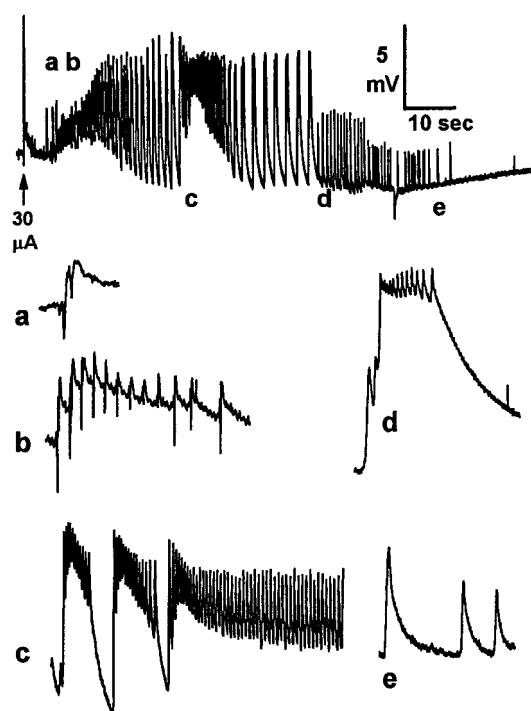


Fig. 8. Ethanol withdrawal induces ictal events in chronically exposed explants. Top trace shows an electrographic seizure recorded in area CA1 evoked by a single shock (30 μ A, arrow) in an explant slice after withdrawal from 35 mM ethanol. Traces labeled a through e show expanded waveforms at points corresponding to letters in top trace. The duration of EGS events were measured from the onset of the first spontaneous burst to the last phasic burst before the quiescent period.

quence with no events of intermediate amplitude or duration observed. For the explant shown in figure 8, four events were measured during the withdrawal period with a mean duration of 93 sec and a range from 85 to 105 sec. In other explants, long-lasting seizures occurred that had a similar morphology except that there was little or no tonic, high-frequency firing phase, and the event consisted of phasic bursts separated by hyperpolarizing shifts. The duration of this type of electrographic seizure was more variable from slice to slice, ranging from 30 sec to several minutes in duration, but again tended to be similar for a particular explant.

The expression of ictal events during ethanol withdrawal was dependent on the activation of NMDA receptors (fig. 9A). Application of D-APV (25 μ M for 35 mM CE slices, $n = 5$; 50 μ M for 75 mM CE slices, $n = 6$) resulted in the complete loss of seizure events in all slices that had previously displayed ictal activity. The amplitude of spontaneous burst events and abrupt depolarizing shifts was also reduced after drug exposure. In several experiments where input/output curves were recorded hourly during the entire recording period, higher stimulus intensities typically evoked a larger slow component. Analysis of the relationship between stimulus intensity, slow PSP amplitude and EGS expression within single explants revealed that EGSs occurred after evoked PSPs that exhibited larger slow components whereas they were never observed after PSPs with smaller components evoked by lower stimulus amplitudes. For the 35 mM CE group, the slow component was $84 \pm 4\%$ of peak for PSPs that evoked EGSs and $19 \pm 8\%$ of peak for PSPs evoked at lower stimulus amplitude where no EGS occurred ($P < .01$, $n = 4$). For the 75

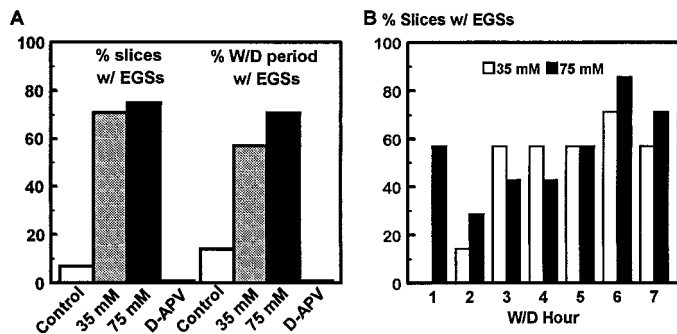


Fig. 9. Ictal events seen during ethanol withdrawal are NMDAR-dependent. A, Histograms show percentage of explant slices in each group that showed ictal activity at some point in the recording period (left), and frequency of ictal events in slices displaying ictal activity (right). Seizure frequency was calculated as the average number of hours in the 7-hr withdrawal period where ictal events were observed for each slice. B, Histogram shows the number of slices in each CE group exhibiting at least one ictal event during each 1-hr period during withdrawal.

mM CE group, these values were $78 \pm 6\%$ of peak and $31 \pm 10\%$ of peak, respectively ($P < .01$, $n = 6$). In 2 explants where recordings were continued after D-APV washout, EGS events were observed only after the slow component of the PSP had reappeared. Therefore, the induction of ictal events was time-locked with the expression of the NMDAR-dependent synaptic component.

Ictal events were observed at nearly equal frequency in slices exposed to 35 mM and 75 mM ethanol and tended to occur more frequently later in the recording period after ethanol withdrawal (fig. 9B). In the 35 mM CE group, EGSs were exhibited in 5 of 7 slices during the 7-hr withdrawal period. On average, these events occurred at least once during 4 of the 7 hr of the withdrawal period. For the 75 mM group, where 6 of 8 slices exhibited EGSs, the events occurred in 5 of the 7 hr on average. No ictal events were observed in ethanol-exposed slices from either group during the prewithdrawal period where ethanol was present in the recording solution. In the control groups, only one slice from

the 35 mM group displayed an ictal event, and this event occurred only once, late in the recording period (*i.e.*, one event detected in over 100 hr of recordings from 10 slices).

NMDAR subunit composition in hippocampal explants. *In situ* hybridization techniques were used to determine whether mRNA coding for NMDAR subtypes known to be present in native hippocampus were present in the explants after 2–3 weeks in culture. Figure 10A shows images of representative autoradiograms from slices incubated with oligonucleotide probes for the NR1, NR2A and NR2B subunit message. The oligonucleotide sequence for the NR1 subunit (pan-R1) was complementary to a sequence encoding amino-acid residues 566–580, common to all splice variants of the NR1 polypeptide (Monyer *et al.*, 1992). Binding was observed for all three probes, primarily in the cell body layer of areas CA1 and CA3 and the granule cell layer of dentate gyrus.

Chronic effects of ethanol on NR2B subunit expression. Alterations in NR2B mRNA and protein expression after chronic ethanol exposure have been reported (Follesa and Ticku, 1996; Hu *et al.*, 1996). Expression of mRNA encoding the NR2B subunit was therefore examined in explants after chronic exposure to either 35 mM or 75 mM ethanol. Two independent sets of experiments were performed for each ethanol concentration. These data are summarized in figure 10B. Exposure to 35 mM ethanol resulted in an average increase in NR2B subunit message to $120 \pm 9\%$ of control ($n = 21$ control, $n = 21$ ethanol slices, both experiments pooled). Exposure to 75 mM ethanol resulted in an increase in NR2B subunit message to $147 \pm 16\%$ on average ($n = 15$ control, $n = 16$ ethanol slices, both experiments pooled). Statistically significant differences were obtained for both CE groups in one of the two independent experiments (35 mM, $P < .05$; 75 mM, $P < .01$) whereas pooling of data from both experiments resulted in no overall significant difference. Thus, there was a trend which suggests that the level of NR2B message was higher in ethanol-exposed explants than in controls, consistent with previous reports of changes in

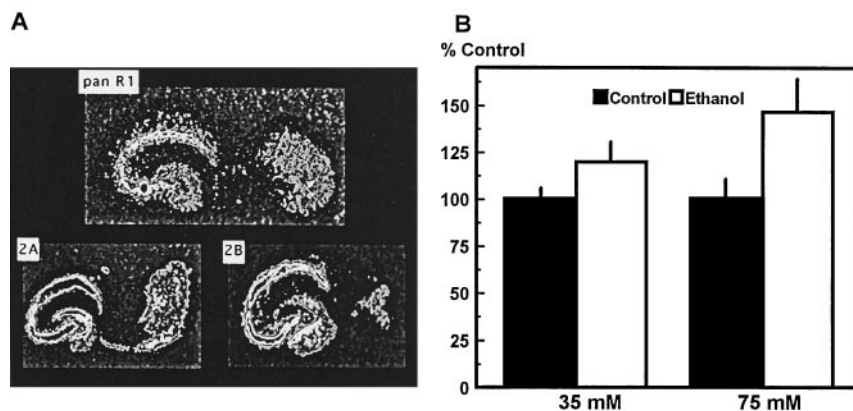


Fig. 10. *In situ* hybridization reveals presence of mRNA for NMDAR subunits. A, Representative autoradiograms showing NR1, NR2A, and NR2B subunit mRNA distribution in explants cultured for 2–3 weeks. Slices incubated with excess cold oligonucleotide probe for the 2B subunit displayed no nonspecific binding (not shown). B, Relative NR2B subunit probe density in CE *vs.* control explants. Values are shown for each concentration of ethanol (35 mM and 75 mM) pooled from two experiments for each group relative to control values. Pooled values for the CE explants were not different from control values for either group (Student's *t* test). *Note:* To control for variations in slice thickness, we established a thickness calibration assay using the coomassie blue protein stain. Native brain tissue was sliced from 10 to 150 (thick using a microtome and thaw-mounted to microscope slides). The tissue slices were treated in the same fashion as the explant slices (*i.e.* fixation, *in situ* rinsing, and dehydration conditions) and then incubated in a 0.001% coomassie blue solution for 1.5 hr, followed by overnight incubation in a destaining solution (45% methanol, 10% glacial acetic acid). Explant tissue from 35 mM study #2 was subjected to the same staining protocol after the *in situ* protocol, and the density of each explant slice was compared to a calibration curve established using the native tissue slices. This procedure did not significantly affect the difference between control and ethanol-exposed tissue.

NR2B subunit mRNA after chronic ethanol exposure (Hu *et al.*, 1996).

Discussion

NMDAR-mediated synaptic responses and the acute effects of ethanol. NMDAR-mediated EPSCs recorded from CA1 pyramidal cells in the explants showed characteristics similar to NMDAR-mediated currents isolated in acute slice preparations (Hestrin *et al.*, 1990; Randall *et al.*, 1990). The mean time course of the EPSCs was similar to that observed in recordings from acute slices from mature rats (Hestrin *et al.*, 1990; Randall *et al.*, 1990). The similarity between the NMDAR-mediated EPSCs recorded in the explants and those recorded in acute slices of hippocampus from mature brain provides additional evidence to suggest that NMDAR function develops normally in the explants (Bahr, 1995).

Prior to examining the effects of chronic ethanol exposure on NMDAR function in the explants, it was important to establish whether this receptor subtype was sensitive to acutely applied ethanol. Ethanol has been shown to inhibit NMDAR-mediated synaptic responses recorded in acute hippocampal slices (Lovinger *et al.*, 1990; Morrisett and Swartzwelder, 1993) and NMDA-induced currents in dissociated neurons (Lovinger *et al.*, 1989). However, recent evidence suggests that the sensitivity of NMDARs to ethanol may decrease with developmental age (Swartzwelder *et al.*, 1995; Lovinger, 1995). With this possible confound in mind, we used a concentration of ethanol (75 mM) demonstrated to maximally inhibit NMDAR-mediated responses in mature hippocampal tissue (Lovinger *et al.*, 1990; Morrisett *et al.*, 1991). The level of inhibition of NMDA EPSCs by 75 mM ethanol acutely applied to the explants was close to that seen for CA1 population synaptic potentials recorded in acute slices from adult rats (Lovinger *et al.*, 1990; Morrisett *et al.*, 1991). Additionally, these results provide the first demonstration of an acute inhibitory effect of ethanol on NMDAR-dependent synaptic currents in hippocampal explants.

Linkage of NMDAR synaptic responses and ictal events. Field recordings demonstrated the selective enhancement of a slow, NMDAR-dependent component of the evoked PSP in explant slices after *in vitro* withdrawal from chronic ethanol exposure. This conclusion regarding the specificity of the enhancement to the NMDA *vs.* the non-NMDA component of transmission is supported by the following evidence. The threshold stimulus amplitude (reflecting pre-synaptic fiber excitability) was unaltered after chronic ethanol exposure and withdrawal. The average peak amplitude of the field PSP, reflecting the non-NMDAR mediated component of excitatory transmission, was not significantly different in ethanol-exposed explants and was unaffected by NMDAR antagonists. Conversely, Molleman and Little (1995), using acute slices prepared from animals chronically exposed to ethanol, have observed increases in isolated fast excitatory synaptic potentials. However, the increases were not observed until several hours after withdrawal, which was taken as the time of slice preparation. There are technical and theoretical reasons which might account for this difference; however, we would suggest that a delayed upregulation of the non-NMDA component may occur as a consequence of an earlier enhanced NMDAR activity during withdrawal.

Our finding of an increase in an NMDAR-mediated synap-

tic component in the hippocampal explants was statistically significant only after withdrawal (however there was a trend for enhancement during chronic 35 mM exposure, possibly reflecting less acute inhibition by ethanol). Indeed, this points up the major thrust of this study: to understand the role of NMDAR function in withdrawal seizure expression. Previous investigators have reported alterations in NMDAR channel antagonist binding or NMDAR-dependent calcium flux in native tissue after chronic exposure but before withdrawal (Grant *et al.*, 1990; Iorio *et al.*, 1992) and in electrophysiological assessment of native tissue prepared from animals after withdrawal (Ripley and Little, 1995; Whittington *et al.*, 1995). The present work extends these findings but is distinct in one important respect: we have electrophysiologically monitored the alterations in NMDAR function during the withdrawal period and correlated the observed changes with electrographic seizure event occurrence. This is a particular technical advantage afforded by the explant model system that enables the linkage of synaptic mechanisms to the pathological phenomenon of interest, with real time assays for both measures.

The most significant finding of this study is the direct correlation between the enhanced NMDAR activity observed after the washout of ethanol and the subsequent expression of electrographic seizures. Several lines of evidence indicate that the expression of the ictal events was dependent on activation of NMDARs. First, the induction of EGS events was temporally correlated with the expression of the NMDAR-dependent component of the field PSP. Thus, EGSs were only initiated after evoked or spontaneous PSPs that exhibited a large slow component. Second, the EGSs were completely abolished in the presence of the NMDAR antagonist, D-APV. Further, the expression of seizure activity coincided with the reappearance of a large slow PSP component during the washout of the receptor antagonist. Third, ictal events were not observed before the withdrawal of ethanol from the recording media, a period where the NMDAR-dependent PSP component was substantially reduced. Therefore, we conclude that NMDAR activation is required for the expression of postwithdrawal ictal activity in hippocampus.

The ability of an *in vitro* model system to exhibit EGS events is of obvious advantage for the study of cellular mechanisms of withdrawal hyperexcitability. However, we recognize that it is important to distinguish between hyperexcitability that may develop due to the effects of chronic ethanol exposure and that described previously in long-term explant preparations. Explants cultured using the roller-tube method (Gähwiler, 1981) have been reported to display an increased level of excitability with increasing time in culture (McBain *et al.*, 1989). These explants display a progressive onset of spontaneous epileptiform bursts and can exhibit long-lasting ictal events, particularly after 30 days in culture (McBain *et al.*, 1989). In the present study, explants were cultured using the interface method (Stoppini *et al.*, 1991) which preserves more three-dimensional structure of the hippocampal network. The slices were maintained *in vitro* for 1 week before exposure to ethanol, a period of time that allowed for recovery of synaptic responses (Muller *et al.*, 1993). Recordings were thus performed on tissue that had been in culture for 2 to 3 weeks, and we observed ictal activity in only 1 of 14 control slices. We conclude that slices cultured using the interface technique display more acceptable characteristics

for a study such as this than slices cultured using the roller-tube technique.

One potential confound is related to the possibility that neurotoxicity may develop due to our exposure and/or withdrawal paradigm, and it is this neurotoxicity that may be responsible for the hyperexcitability observed. We have assessed this possibility in another extensive study through the use of propidium iodide fluorescence techniques (Thomas and Morrisett, 1997; Thomas and Morrisett, submitted). In that study, we found no evidence for a role of neurotoxicity mechanisms in the induction or expression of the withdrawal hyperexcitability. Furthermore, those studies substantiated the present results in which increased NMDA receptor function was observed after withdrawal from chronic ethanol exposure.

NMDAR subunit composition and the effects of chronic ethanol exposure. NMDA receptor ionophores are thought to exist as heteromeric complexes consisting of subunits derived from two related gene families, designated NMDAR1 (or NR1) and NMDAR2 (NR2) for rat brain (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992). Developmental changes in subunit expression have been described for rodent brain (Watanabe *et al.*, 1992; Monyer *et al.*, 1994; Sheng *et al.*, 1994; Wenzel *et al.*, 1997). In particular, NR2A subunit expression in hippocampus is low at birth and peaks during the first 3 postnatal weeks (Wenzel *et al.*, 1997), whereas NR2B expression peaks perinatally before declining to adult levels (Watanabe *et al.*, 1992). The presence of both NR2A and NR2B subunit mRNA in explants cultured for several weeks is thus consistent with the maintenance of a relatively mature NMDAR subunit composition in organotypic hippocampal cultures.

Changes in NMDAR subunit expression have been reported after chronic ethanol exposure (Trevisan *et al.*, 1994; Hu *et al.*, 1996). Hu and coworkers (1996), using an RNase protection assay, reported that in cultured dissociated cortical neurons after chronic ethanol exposure the expression of mRNA encoding the NR2B subunit was selectively increased. We observed an increase in NR2B subunit mRNA levels in the explants after chronic ethanol exposure using *in situ* hybridization techniques, consistent with the results of Hu *et al.* (1996). These data thus support the hypothesis that an increase in NR2B mRNA expression occurs after exposure of central neurons to chronic ethanol and may represent one mechanism contributing to the observed enhancement of NMDAR function.

Summary. We have demonstrated a marked enhancement of NMDAR function in hippocampal explants which is apparent after withdrawal from chronic (but not acute) ethanol exposure, which is also strongly correlated with the expression of NMDAR-dependent epileptiform events. These findings appear to satisfy criteria that support a causative relationship between enhancement of NMDAR function and the induction and subsequent expression of ethanol withdrawal hyperexcitability. The primary caveat to this argument is that it assumes that the expression of withdrawal hyperexcitability exhibited by the explanted hippocampus represents a model system that is relevant to behavioral seizure expression during ethanol withdrawal. Obviously, other brain regions, most notably the inferior colliculus (McCown *et al.*, 1995), also represent important sites for withdrawal hyperexcitability.

The role that this alteration in NMDAR function plays in the generation of the withdrawal events in the whole animal in terms of the generation of the paroxysmal depolarizing shift remains to be explored, particularly with respect to other cellular alterations contributing to withdrawal hyperexcitability. Taken together, these different threads should help begin to tie together a more cogent and thorough understanding of the alterations in NMDAR function which may underlie ethanol withdrawal hyperexcitability and seizures.

Acknowledgments

The authors would like to thank Dr. Ben Bahr for technical advice on culturing hippocampal explants and Dr. Pat Randall for assistance with the statistical analyses.

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