ETHANOL ALTERS THE FREQUENCY, AMPLITUDE AND DECAY KINETICS OF Sr²⁺-SUPPORTED, ASYNCHRONOUS NMDAR mEPSCs IN RAT HIPPOCAMPAL SLICES

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Abstract

To discriminate between pre- and post-synaptic effects of ethanol on NMDAR-signaling in hippocampus, we adapted the technique of Sr$^{2+}$-substitution to the hippocampal blind slice patch-clamp preparation. Hippocampal slices were isolated from 12-20 day old rats sacrificed in accordance with University of Texas IACUC guidelines. NMDAR mEPSCs were evoked from CA1 pyramidal neurons in the presence of Sr$^{2+}$ (4 mM), causing the synchronous EPSC observed in the presence of Ca$^{2+}$ to be supplanted by asynchronous mEPSCs. Amplitudes typically ranged from 5-40 pA and responded to the NMDAR antagonist (DL)-APV (50 $\mu$M) with a statistically significant reduction in mean amplitude. Ethanol (25, 50, and 75 mM) exerted dose-dependent effects on mEPSC amplitude and frequency. Peak amplitude inhibition was observed at 75 mM ethanol. Notably, ethanol significantly decreased event frequency at 50 and 75 mM ethanol. Ethanol (75 mM) also significantly increased the paired-pulse ratio of NMDAR EPSCs. Cumulative comparisons of decay time constants derived from single-exponential fitting of mEPSCs revealed significantly accelerated current decay kinetics in the presence of 75 mM ethanol. Taken together, these reductions in miniature event frequency and amplitude, concurrent with an increased rate of decay, suggest that the acute effects of ethanol on NMDAR signaling at hippocampal synapses are multifocal in nature. This finding of pre-and post-synaptic effects of ethanol on NMDAR signal strength in a brain region central to cognition is wholly consistent with previous reports of ethanol inhibition of NMDAR-LTP in vitro and with the profound cognitive deficits associated with binge-level intoxication in vivo.
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Introduction

NMDA receptors (NMDARs) are a major substrate for a variety of neurophysiological and neuropathological processes due to their widespread CNS expression and unique functional characteristics. Ethanol has complex effects on NMDAR-dependent synaptic transmission due to its direct actions on NMDARs (Hoffman et al., 1989; Lovinger et al., 1989, 1990) as well as on voltage-gated Ca\(^{2+}\) channels (VGCCs) in various preparations including synaptosomes (Harris and Hood, 1980) and brain slices (Whittington and Little, 1991, 1993; Hendricson et al., 2003). Ethanol prevents the formation of NMDAR-dependent LTP in hippocampal slices from mature rats (Durand and Carlen, 1984; Blitzer et al., 1990; Morrisett and Swartzwelder, 1993) and facilitates the induction of NMDAR-dependent LTD in young rats, potentially via an NR2B subunit-dependent mechanism (Hendricson et al., 2002).

In addition to these effects on synaptic mechanisms underlying cognition, extensive evidence indicates that the antagonist effect of ethanol on NMDARs is closely associated with the neurochemical alterations underlying withdrawal hyperexcitability and subsequent neurotoxicity (Grant et al. 1990; Morrisett et al., 1990; Chandler et al., 1993; Ripley and Little, 1995; Blevins et al., 1995; Thomas et al., 1998; Thomas and Morrisett, 2000). Ethanol also exerts complex actions on neural pathways of reward via interactions with NMDAR signaling. For instance, a recent report from our lab indicates that the ethanol sensitivity of NMDARs in the nucleus accumbens is modulated by the activation of a D1 receptor / cAMP / PKA / DARPP-32—dependent pathway (Maldve et al., 2002).
Despite the quantity of information relating ethanol effects on NMDAR-mediated neurotransmission to neurocognitive and neurophysiological deficits associated with acute and chronic ethanol intoxication, dependence formation, and withdrawal, detailed miniature synaptic analysis of ethanol effects on the amplitude and frequency of NMDAR miniature EPSCs (mEPSCs) in isolation has not been performed. Methodological considerations may play a role in this omission: NMDAR mEPSCs characteristically exhibit low intrinsic frequency, slow rise times, and small amplitudes (Ozawa et al., 1998). This combination of attributes makes mEPSCs arising in the presence of tetrodotoxin to block action potential conduction ill-suited for frequency analysis, as the construction of cumulative occurrence histograms requires more events than can typically be recorded in the duration of a standard whole cell patch clamp recording.

Previous studies from our lab (Morrisett and Swartzwelder, 1993; Thomas et al., 1998) using the hippocampal blind slice patch clamping preparation have examined the effects of a high concentration of ethanol (75 mM) on synaptically evoked NMDAR EPSCs and reported pronounced reductions in mean current amplitude. However, event frequency analysis was not performed in those studies. The dearth of direct electrophysiological measures of ethanol effects on presynaptic function is highly significant, especially in light of the well-developed literature describing the inhibitory effects of ethanol on VGCCs. A recent study from our lab employing confocal microscopy with the lipophillic dye FM1-43 reported that in the field CA1 region of intact hippocampal slices, ethanol acts to inhibit vesicular release evoked by high-K⁺ depolarization of nerve terminals via an action on N/P/Q-VGCCs (Maldve et al., in press). This study also contains
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further electrophysiological evidence for presynaptic actions of ethanol in the hippocampus, as we demonstrate that ethanol inhibits the extent to which elevated extracellular K⁺ increases the frequency of AMPAR mEPSCs recorded from CA1 pyramidal neurons.

To directly address the issue of the effect of ethanol on presynaptic function while maintaining the advantages afforded by the use of synaptic stimulation, we optimized event characteristics by replacing Ca²⁺ in recording solutions with Sr²⁺, which readily traverses VGCCs and supports quantal transmitter release (Miledi, 1966). While release supported by Ca²⁺ is characterized by a monotonically decrementing population of quanta, release supported by Sr²⁺ is asynchronous in nature, consisting of multiple quantal synaptic events (Dodge et al., 1969). Though Sr²⁺-substitution has been employed in multiple brain regions to facilitate the analysis of changes in AMPAR (Oliet et al., 1996; Choi and Lovinger, 1997) and GABAR (Morishita and Alger, 1997; Caillard et al., 1999) signaling associated with synaptic plasticity, we are aware of only one report employing Sr²⁺-substitution in a study of NMDAR currents (Umemiya et al., 2001). However, this report focused upon event kinetics and did not utilize frequency and amplitude analysis.

The use of Sr²⁺-substitution to augment mEPSC yield confers certain methodological advantages compared with other techniques used in the analysis of NMDAR mEPSCs. Analysis of asynchronous events evoked in Sr²⁺ circumvents the limitations of analysis of spontaneous events occurring in tetrodotoxin (TTX), since asynchronous events are synaptically evoked, thereby ensuring an adequate event yield and yet remain non-action potential dependent.
Other studies have employed such techniques as recording in Mg\(^{2+}\)-free solutions (Myme et al., 2003) or recording in the presence of elevated extracellular K\(^+\) to induce sustained depolarization of nerve terminals (Ziskind-Conhaim et al., 2003). The use of Sr\(^{2+}\)-substitution represents an improvement over these methods as it allows for the recording of mEPSCs in the presence of only slightly reduced extracellular Mg\(^{2+}\), limiting the incidence of NMDAR “flickering” behavior common in Mg\(^{2+}\)-depleted solutions (a phenomena which complicates analysis by making mEPSCs less discrete). Additionally, in the present configuration events are recorded in the absence of sustained elevations in synaptic and extra-synaptic glutamate induced by recording in elevated extracellular K\(^+\), a setting which more closely approximates *in vivo* conditions.
Methods

Slice Preparation Hippocampal slices were prepared from Sprague-Dawley rat pups of both sexes (12-20 days old) using previously described techniques (Morrisett and Swartzwelder, 1993; Thomas et al. 1998) which are in accordance with NIH and University of Texas IACUC guidelines. Briefly, hippocampi were removed bilaterally and 500 micron transverse sections were cut, transferred to a holding chamber containing ACSF bubbled with 95% O\textsubscript{2} / 5% CO\textsubscript{2} (carbogen), and maintained at 32° C for a minimum of 60 min prior to recording. ACSF consisted of (in mM): NaCl, 120; NaHCO\textsubscript{3}, 25; KCl, 3.3; NaH\textsubscript{2}PO\textsubscript{4}, 1.2; CaCl\textsubscript{2}, 1.8; MgSO\textsubscript{4}, 2.4; dextrose, 10.

Patch-clamp Recording Tight-seal whole-cell patch recordings were made at 32°C from CA1 pyramidal neurons as previously described (Morrisett and Swartzwelder, 1993). Recording electrodes were made from thin-walled borosilicate glass (TW150F-4, WPI, Sarasota, FL, 1.2-2.2 M) and filled with (in mM): CsF, 115; NaCl, 12; EGTA, 0.5; HEPES, 10; Mg\textsuperscript{2+}-ATP, 2; Tris-GTP, 0.3; QX-314, 20; 280-290 mOsm, pH 7.2 with CsOH. EPSCs were evoked by stimulation of Schaeffer collateral fibers in the stratum radiatum layer of area CA3 via monopolar tungsten electrodes (WPI). Constant-current pulses (100 µsec duration, 10-40 µA amplitude) were applied through a stimulus isolation unit driven by an analog stimulator (WPI). The NMDAR-mediated component of synaptic current was recorded after pharmacological elimination of other major synaptic components: Fast, non-NMDAR-mediated mEPSCs were blocked by bath application of the AMPA/KA antagonist, DNQX (10 µM; Sigma Chemical
Co., St. Louis, MO). GABA currents were blocked with picrotoxin (25 µM; Sigma). Activation of NMDARs was facilitated by lowering extracellular Mg$^{2+}$ to 0.6 mM (from a standard recording solution concentration of 0.9 mM) during patch-clamp recording and by holding cells at slightly depolarized membrane potentials (-45 to -55 mV) to partially alleviate Mg$^{2+}$ blockade of NMDARs. To support asynchronous release, Sr$^{2+}$ (4 mM) supplanted Ca$^{2+}$ in recording solutions.

Asynchronous events typically originated within 500 ms of the synaptic stimulus and persisted for 3-5 seconds thereafter. In a typical experiment, 25-30 stimuli were delivered at a frequency of .075 Hz followed by a 6-8 minute drug wash-in period. Recordings in the presence of ethanol and APV were initiated immediately following drug wash-in. Data were acquired for a period of 6 seconds following each stimulus. Due to the marked inhibition of event frequency observed in the presence of 50 and 75 mM ethanol, synaptic stimuli were delivered at the above frequency for up to an additional 5 minutes to augment event yield in the face of decreased release probability. In paired-pulse facilitation experiments, a train of 2 identical constant-current pulses (100 µsec duration, 10-40 µA amplitude; see above) with inter-stimulus intervals of 50 or 100 ms was delivered to the Schaeffer collateral pathway once every 30 seconds; typical experiments contained 5-10 responses per experimental condition. As in recordings of Sr$^{2+}$-supported events, cells were held at slightly depolarized (-45 to -55 mV) membrane potentials. The recording solution employed in paired-pulse facilitation experiments contained 2 mM Ca$^{2+}$ and was identical to that described above in all other respects.
Data Acquisition and Analysis  Recordings were made using an Axoscope 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, digitized at 10-20 kHz via an Axon Digidata 1200 interface board and stored on hard media for subsequent offline analysis under a Windows XP environment. Recordings in which access resistance changed more than 10% during the course of the experiment, or changed in such a way that could explain changes in mEPSC characteristics associated with drug administration, were discarded. Cells which maintained stable electrophysiological characteristics throughout the recording period were screened for inclusion in cumulative analyses of drug-induced effects by the construction of single-cell cumulative frequency histograms with subsequent Kolomogorov-Smirnov (K-S) statistical testing using the MiniAnalysis software suite (v. 5.01; Synaptosoft, Decatur, GA). Less than 10% of total cells were omitted from subsequent analyses based on this criterion. Intermittent noise spikes were removed manually and via the inclusion of an area threshold criterion in the analysis parameters. Recordings exhibiting persistent noise interference were not included in cumulative analyses.

In cumulative analysis of amplitude, inter-event intervals, and decay time constants, all events for a given condition exceeding a minimum amplitude threshold (typically 5-7 pA) were included in analyses; the amplitude threshold was always held constant within cells. For the 23 cells in the ethanol group, the average number of mEPSCs per cell in the control condition was 171, and the average number in the presence of ethanol was 134. Event totals for each concentration of ethanol were as follows: 25 mM ethanol, 1251 (control) / 1223 (ethanol). 50 mM ethanol, 1062 / 713. 75 mM ethanol, 1626 / 1151. For the 4
cells in the APV group, the average number of mEPSCs in the control condition was 160, with an average of 74 following drug administration. The total number of events in the control condition was 641, and the total number of events in the presence of APV was 294.

Percent changes in mean event amplitude and frequency following drug application were assessed via Student’s t-test (p values < 0.05 were considered statistically significant). The time constant for the decay of mEPSCs was determined by fitting the decay to a single exponential. In experiments examining the effects of ethanol on PPF of NMDAR EPSCs, the peak amplitude of the first EPSC was subtracted from that of the second EPSC and the percent facilitation of paired-pulse ratio (PPR) was expressed as the product of the following formula: \[(EPSC2-EPSC1) / EPSC1 \] x 100. In all figures subsequent to Figure 1, the portion of raw data traces containing the stimulus artifact has been omitted for clarity.
Results

Characteristics of Asynchronous NMDAR mEPSCs

Substitution of Sr\(^{2+}\) (4 mM) for Ca\(^{2+}\) in the recording ACSF eliminated the large (typical amplitudes ranging from 50-125 pA) EPSC response to synaptic stimulation typical of this preparation under standard (i.e. in the presence of extracellular Ca\(^{2+}\)) recording conditions. In the place of this synchronous synaptic response, multiple discrete mEPSCs originating within 500 ms of the synaptic stimulus and persisting for several seconds were repeatably observed. Typical amplitudes ranged from 5-40 pA. The mean mEPSC amplitude for all cells prior to drug application was 12.0 ± 4.5 pA (n = 4580 events from 27 cells).

As shown in Figure 1, the Sr\(^{2+}\)-supported asynchronous response to synaptic stimulation was accompanied by a substantial leftward shift in the cumulative inter-event interval (IEI) distribution of a representative cell. By convention, such shifts are indicative of increased event frequency. Asynchronous mEPSCs exhibited the slow kinetics of rise and decay characteristic of NMDAR miniature currents: The mean rise time, averaged across all cells in the ethanol group (n=23) prior to drug exposure, was 19.2 ± 2.7 ms, and the mean decay time constant (\(\tau\)), derived from a single exponential function, was 102.4 ± 9.0 ms.

For the present study, events were evoked in the presence of the AMPA/KA receptor blocker DNQX, as well as under conditions optimized to favor the occurrence of NMDAR-mediated events (reduced extracellular Mg\(^{2+}\) and slightly depolarized membrane potentials; see Methods). As stated, these mEPSCs exhibited the slow kinetics and moderate amplitudes commonly
associated with spontaneous NMDAR currents. To further confirm the pharmacological identity of this population of events, we employed the NMDAR-selective antagonist (DL)-APV at a standard concentration (50 µM). Bath application of this drug strongly and reversibly inhibited the amplitude and frequency of evoked NMDAR mEPSCs; Figure 2 depicts data from a representative cell. In the presence of APV, mean mEPSC amplitude decreased to a value only slightly greater than the threshold for event detection (38.8 ± 2.8 % of control, p < 0.03; n=5). Figure 7 depicts cumulative statistical analysis of all drug conditions.

Effects of Ethanol on mEPSC Amplitude and Inter-event Interval

Our lab and others have previously demonstrated the antagonism of NMDAR EPSCs by ethanol (Hoffman et al., 1989; Lovinger et al., 1989, 1990; Morrisett and Swartzwelder, 1993). However, as a result of the methodological considerations described previously, these studies did not address the effects of acute ethanol administration on quantal event parameters. Using Sr²⁺ substitution to augment the frequency of quantal events, we constructed cumulative amplitude and frequency histograms of NMDAR mEPSCs to detect the effects of acutely administered ethanol on these measures.

Figure 3 depicts raw event data and cumulative amplitude and IEI histograms from a representative cell acutely exposed to 25 mM ethanol. No statistically significant changes in mean event amplitude (94.8 ± 3.8 % of control, p = 0.6) or IEI (36.8 ± 18.9 % increase in mean IEI; p = 0.2) were induced by ethanol at this concentration. Cumulative amplitude and IEI data for all
concentrations of ethanol are shown in Figure 6. Figure 4 depicts the effects of 50 mM ethanol on a representative cell. In the presence of 50 mM ethanol, mean mEPSC amplitude was reduced to 84.4 ± 4.3 % of control; however, this reduction in amplitude was not statistically significant (p = 0.3; n=5). Notably, at this concentration of ethanol the mean IEI increased by 185.6 ± 63.5 % relative to control (p < 0.05), a finding indicative of a significant decrease in mEPSC frequency at this concentration of ethanol. Figure 5 depicts findings from a representative cell from the 75 mM ethanol group. At this concentration, both mean amplitude (77.6 ± 1.5 % of control, p < 0.03; n=10) and IEI (increase of 149.3 ± 31.2 % relative to control, p < 0.003) differed significantly from the control condition. Amplitude inhibition at 75 mM ethanol also differed significantly from the level of inhibition observed at 25 mM ethanol (p < 0.005).

While the mean increase in IEI observed at 75 mM ethanol was 36.3% less than the increase in this measure observed at 50 mM, the difference between the two data sets was not statistically significant (p = 0.5) though both data sets differed significantly from control. This finding indicates that the effect of ethanol to inhibit event frequency may saturate at approximately 50 mM ethanol. In summary, ethanol significantly inhibited mean NMDAR mEPSC amplitude and frequency at 75 mM, and significantly inhibited event frequency at 50 mM. The effects of 25 mM or 50 mM ethanol on the amplitude measure were not statistically significant.
Effects of Ethanol on Paired-Pulse Facilitation of NMDAR EPSCs

As described above, ethanol exerted a significant effect on the mean IEI of NMDAR mEPSCs, a finding strongly suggestive of a presynaptic effect of ethanol on transmitter release. To further investigate our hypothesis that this effect was a direct consequence of a presynaptic effect of ethanol to suppress synaptically-evoked transmitter release—as opposed to a false positive caused by postsynaptic decreases in NMDAR open probability—we examined the effect of ethanol on paired-pulse facilitation (PPF) of NMDAR EPSCs. PPF describes the phenomena, originally described by Del Castillo and Katz (1954), whereby the second of 2 synaptic stimuli delivered in rapid (typically, 20-200 ms) succession will be larger than the first due to transient presynaptic Ca$^{2+}$ loading. This paradigm is used as an index of drug or synaptic plasticity-induced changes in pre-synaptic function based on the observation that decreasing the extracellular Mg$^{2+}$ / Ca$^{2+}$ ratio, a manipulation which increases release probability in a number of brain preparations including hippocampus (Manabe et al., 1993), is reliably associated with a decrease in PPF.

By convention, paired-pulse ratio (PPR; calculated as Resp 2 - Resp 1 / Resp 1) is inversely proportional to the release probability at a given synapse, such that an increase in PPR indicates a decrease in release probability. Figure 7 depicts the results of experiments testing the effects of 75 mM ethanol on PPF of NMDAR EPSCs evoked by Schaeffer collateral stimulation at 2 inter-stimulus intervals (ISIs). The mean PPR in the control condition at an ISI of 50 ms was 1.0 ± 0.2 (n=6). Following bath application of 75 mM ethanol, the mean PPR increased by 89.0 ± 41.7 % relative to control (p < 0.05). When the ISI was
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extended to 100 ms, the baseline PPR was 0.8 ± 0.1; n=4. After 75 mM ethanol application, the PPR similarly increased by 102.5 ± 12.5 % relative to control (p < 0.03). The difference between the baseline values for the 2 stimulus intervals was not statistically significant (p = 0.5).

**Effects of Ethanol on mEPSC Decay Kinetics**

The effect of ethanol on NMDARs is delimited to a certain extent by receptor NR2 subunit composition with NR2B subunit-containing receptors exhibiting enhanced sensitivity to ethanol inhibition (Lovinger et al., 1995). Receptors containing NR2B also display slower kinetics of activation and inactivation than NR2A-containing receptors (Monyer et al., 1994; Chen et al., 1999). The neonatal rat hippocampus robustly expresses NR2B and NR2D-containing NMDARs from the early post-natal period (Monyer et al., 1994; Kirson et al., 1999), while NR2A expression in this region is initially fairly low, then increases markedly through the second and third post-natal weeks (Zhong et al., 1995).

Based on these observations and given that the age range (12-20 do) of the animals used in this study coincides temporally with the developmental upswing in hippocampal NR2A subunit expression, we sought to examine whether ethanol antagonism of NR2B-containing receptors would alter NMDAR mEPSC kinetics. To measure the effects of ethanol on decay kinetics of mEPSCs, individual events from a given cell were aligned by their onset and averaged, then the decay of this averaged EPSC prior to and following ethanol administration was fitted to a single exponential function.
Interestingly, mEPSCs recorded in the presence of 75 mM ethanol displayed significantly accelerated kinetics of decay, as indicated by a 32.0 ± 6.3 % decrease in the mean value of the first order decay time constant (97.6 ± 16.9 ms vs. 64.6 ± 10.2 ms; p < 0.03, n=10). Figure 8 A depicts averaged mEPSCs from a representative cell in which the peak current in ethanol has been scaled to the control response (right-hand panel) to highlight the increase in decay slope in the presence of ethanol. This effect on mEPSC decay was not apparent in the 25 or 50 mM ethanol groups. Mean 10-90% rise time also decreased slightly at 75 mM ethanol (16.4 ± 3.9 ms vs. 14.5 ± 3.1 ms); however, this difference was not statistically significant (p = 0.7). Cumulative statistical analysis of changes in mean decay time constants at the 3 concentrations of ethanol tested are presented in Figure 8 B.
Discussion

The pharmacological nature of ethanol interactions with native and recombinant NMDARs has been extensively described, as discussed previously. The present study supports previous findings regarding the antagonist action of ethanol on the magnitude of NMDAR-mediated EPSCs and EPSPs, in particular work from our lab in hippocampus and hippocampal tissue culture (Morrisett et al., 1990, 1991; Morrisett and Swartzwelder, 1993; Thomas et al., 1998). In the present study, a statistically significant reduction in event amplitude was attained at 75 mM. Some previous studies, such as that by Li et al (2002) have reported a greater sensitivity of NMDARs to ethanol, as evidenced by appreciable levels of inhibition of NMDAR EPSCs at concentrations in the 10-30 mM range.

The disparity between that study and the present one likely proceeds from inherent differences in measuring ethanol-induced changes in the amplitude of individual evoked EPSCs or EPSPs vs. quantifying the same effect in a heterogeneous distribution of mEPSCs numbering into the hundreds of events per cell. Additionally, the present experiments were performed under conditions designed to reduce the inhibitory effects of extracellular Mg$^{2+}$ on NMDARs, namely, mildly depolarized holding potentials (-45 to -55 mV) and recording ACSF containing 0.6 mM added Mg$^{2+}$ rather than the standard 0.9 mM. Previous work from our lab has shown that the inhibitory effect of ethanol at NMDARs is reduced under these conditions (Morrisett et al., 1990, 1991).

We also provide good evidence of an inhibitory effect of behaviorally relevant concentrations of ethanol on the frequency of synaptically evoked asynchronous NMDAR mEPSCs, based on statistically significant increases in
mean IEIs in the presence of 50 and 75 mM ethanol and on a statistically significant increase in PPF of NMDAR EPSCs in the presence of 75 mM ethanol. The increase in PPR following ethanol administration indicates that ethanol exerts an inhibitory effect on the probability of synaptically-evoked glutamate release. This finding is in very close accord with the results of mEPSC frequency analysis and taken together the two findings are concrete evidence of a novel inhibitory presynaptic effect of ethanol in hippocampus. The similarity in the effects of ethanol on PPF at stimulus intervals of 50 and 100 ms indicates that this effect of ethanol is independent of ISI.

The most parsimonious explanation for the present effects of ethanol on IEI and PPR is an inhibitory action of ethanol on one or more components of vesicular transmitter release. As previously described, we have recently demonstrated direct effects of ethanol on VGCCs to inhibit evoked vesicular release in intact hippocampal slices using FM1-43 (Maldve et al., in press). These findings directly support the present data which indicates that ethanol exerts an inhibitory presynaptic effect on evoked transmitter release.

In another recent study from our lab examining the effects of ethanol on VGCC-dependent LTP (Hendricson et al., 2003), we report that ethanol has no effect on the frequency of AMPAR mEPSCs arising in CA1 pyramidal neurons. This disparity with the present study employing analysis of evoked EPSCs and mEPSCs suggests that the inhibitory presynaptic effect of ethanol may be selective for evoked transmitter release. Notably, the existence of a functionally distinct vesicular pool selectively associated with evoked release has been suggested by Koenig and Ikeda (1999), and Yoshihara et al (2000) described an
extracellular Ca\(^{2+}\)-independent pathway for the modulation of unstimulated transmitter release at the *Drosophila* neuromuscular junction.

Those findings raise the possibility that distinct transmitter release pathways underlie the evoked NMDAR EPSCs and mEPSCs in the present study vs. the spontaneous AMPAR mEPSCs described in the earlier study from our lab, and that this distinction is likewise responsible for the disparity in presynaptic ethanol effects between the 2 studies. An inhibitory effect of ethanol on evoked transmitter release is also consistent with the well-documented antagonist action of ethanol at VGCCs (Harris and Hood, 1980; Leslie et al., 1983, Wang et al., 1993; Hendricson et al., 2003) and with our recent FM1-43 study which indicates that ethanol inhibits evoked transmitter release via an action on VGCCs.

Though the presently available data indicate a central role for the inhibition of VGCC conductances supporting exocytosis in the inhibitory presynaptic effect of ethanol, intra-cellular factors regulating transmitter release may also play a role in this effect. Recent reports suggest that ethanol exerts a modulatory effect on signaling pathways regulating intracellular Ca\(^{2+}\) release (Gruol et al., 1997) as well as serine/threonine phosphorylation events mediated by PKA (Maldve et al., 2002) or PKC (Proctor et al., 2003). Browning’s group has reported that ethanol affects the tyrosine phosphorylation of dendritic NMDARs (Alvestad et al., 2003). This interesting finding raises the possibility that tyrosine kinase-sensitive components of transmitter release may be susceptible to modulation by ethanol.

It should not escape mention that the NMDAR antagonist APV caused a significant increase in IEI as well. Though NMDARs function as presynaptic autoreceptors at spinal nociceptive synapses (Liu et al., 1994, 1997), control of
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transmitter release by pre-synaptic NMDARs has not been documented in hippocampus. Thus, it is likely that the effect of APV on IEI is due in large part to amplitude reductions in mEPSCs such that a significant portion of events which exceeded the minimum detection threshold in the control condition no longer did so in the presence of this antagonist.

Could the effects of ethanol on IEI be attributed to a similar phenomena? Multiple factors argue against this possibility in the present case. First, complete antagonists such as APV give a false positive against frequency by causing a profound decrease in receptor open probability; however, ethanol is not a complete antagonist at NMDARs, and therefore this confound is highly unlikely. Furthermore, ethanol significantly increased the mean PPR at the synapse under investigation, directly indicative of an ethanol-induced decrease in synaptically-evoked transmitter release probability. Additionally, the magnitude of the observed effects of ethanol on amplitude and IEI is markedly dissimilar—i.e., at 50 mM ethanol, a statistically significant increase in mean IEI was achieved in the absence of a statistically significant reduction in mean amplitude. This departure from APV experiments (in which statistical significance was achieved for both measures and in which amplitude depression was profound) further suggest that ethanol-induced reductions in mean mEPSC amplitude cannot be responsible for such a decrease in event frequency.

Finally, if a decrease in mEPSC amplitude were solely responsible for the observed increases in IEI, then it would be expected that an increase in the overall level of amplitude inhibition by ethanol would cause a proportional increase in IEI. This is not the case in the present experiments, as the increase
in amplitude inhibition observed between 50 and 75 mM ethanol is accompanied by a decrease in the extent to which mean IEI is prolonged. Thus we are confident that the observed increases in IEI in the presence of ethanol are indicative of decreases in mEPSC frequency resulting from presynaptic actions of ethanol.

The presently described effect of ethanol on presynaptic function at CA1-Schaeffer collateral synapses--statistically significant increases in cumulative IEI as well as PPR--is previously undocumented in a native system. Marszalec et al (1998) reported ethanol inhibition of the frequency of mixed AMPAR and NMDAR sEPSCs in dissociated cultured cortical neurons. However, the synaptic structure between inhibitory and excitatory elements is lost in that preparation, making such studies difficult to interpret. Also, that study reported analysis of spontaneous events occurring in the absence of TTX. Spontaneous events cannot be used to discriminate between pre- vs. post-synaptic changes. Finally, that study was performed in the absence of extracellular Mg\(^{2+}\). For these reasons, the present study which circumvents these significant confounds by employing the intact neural network of the hippocampal slice, non-spike driven events and only slightly reduced extracellular Mg\(^{2+}\) represents a more reliable model system for assessing the presynaptic effects of ethanol.

We also report ethanol-induced increases in NMDAR current decay kinetics. One explanation for this effect may be a “biasing” of the total population of synaptic NMDAR currents towards faster-decaying events gated by NR2A-containing receptors in the setting of a selective reduction of slower decaying NR2B-mediated currents by significant concentrations of ethanol. Though this
hypothesis is attractive given the developmental “spike” in hippocampal NR2A expression over an age range (post-natal weeks 2 and 3) nearly identical to the range (post-natal days 12-20) employed for these experiments, it is nonetheless important to take into account the contributions of other NR2 subunit populations, as well.

NR2C expression is also pronounced in the neonatal hippocampus (Hrabetova et al., 2000). While NR2C-containing receptors exhibit lower single-channel conductance than both NR2A and NR2B receptors, their rise and decay kinetics, as determined in expression systems, are similar to NR2B-containing receptors (Monyer et al., 1994). Thus, though faster decay kinetics mediated by NR2A-containing receptors may be more evident during ethanol antagonism of the NR2B current component, this effect may be tempered somewhat by slower NR2C receptor decay kinetics. This “counterbalancing” effect may be the reason that the present facilitation of NMDAR decay kinetics by ethanol was only observed at the highest concentration tested.

The data presented here are indicative of 3 distinct modes of ethanol antagonism of NMDAR-mediated neurotransmission—presynaptically, to decrease event frequency, and post-synaptically, to both decrease event amplitude and accelerate event decay (smaller events which are fewer in number per unit time and dissipate more rapidly). This effect of ethanol on NMDAR-mediated event frequency has particular relevance for understanding the mechanism(s) by which acutely administered ethanol inhibits hippocampal NMDAR-LTP (Durand and Carlen, 1984; Blitzer et al., 1990; Morrisett and Swartzwelder, 1993), enhances LTD following acute administration (Hendricson
et al., 2002), and suppresses LTD following chronic administration (Thinschmidt et al., 2003).

While reductions in NMDAR current amplitude could well be envisioned to degrade the “coincidence detection” function of NMDARs in mechanisms of synaptic strengthening, it is interesting to note that variation of the frequency and duration of tetanizing trains used to elicit LTP \textit{in vitro} has demonstrable effects on the duration of the ensuing synaptic potentiation (Berry et al., 1989; Keller et al., 1991). In light of these observations, it is conceivable that the \textit{in vivo} inhibition of LTP induction by ethanol may be due to the combined effects of concurrent reductions in the frequency and amplitude of NMDAR mEPSCs at central synapses. By extension, the increased kinetics of current decay in the presence of ethanol observed here may also diminish LTP induction.

Taken as a whole, our findings suggest that a comprehensive model of acute ethanol effects at glutamatergic synapses should encompass pre- and post-synaptic sites of action. We envision that an accounting of presynaptic ethanol effects will be useful in describing the changes in glutamatergic signaling which are induced by chronic ethanol exposure and subsequent withdrawal. Furthermore, the use of the novel technique of \textit{Sr}^{2+}-substitution represents a methodological advance in the study of NMDAR-mediated neurotransmission generally and ethanol-NMDAR interactions in particular.
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Ethanol Effects on Asynchronous NMDAR mEPSCs

Works Cited


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Figure Legends

Figure 1. Substitution of Sr\(^{2+}\) (4 mM) for Ca\(^{2+}\) in the recording ACSF reduces the synchronous EPSC response to Schaeffer collateral stimulation and induces asynchronous NMDAR mEPSCs in voltage-clamped CA1 pyramidal neurons from acute hippocampal slices. A. Evoked NMDAR-mediated synaptic currents recorded in the presence of Ca\(^{2+}\). Arrow indicates large synchronous EPSC response to synaptic stimulation (the amplitude of stimulus artifacts have been truncated). Note relative lack of miniature currents in the subsequent recording period. B. Evoked NMDAR-mediated synaptic currents recorded in the presence of Sr\(^{2+}\). Note the reduction in amplitude of synchronous synaptic response and the presence of multiple, discrete mEPSCs in subsequent recording period. C. Cumulative histogram depicting changes in inter-event intervals from 2 cells exposed initially to Ca\(^{2+}\)-ACSF, then superfused with Sr\(^{2+}\)-ACSF.

Figure 2. The NMDAR antagonist (DL)-APV reversibly suppresses amplitude and frequency of pharmacologically isolated, asynchronous NMDAR mEPSCs. A. Raw data from a representative cell prior to drug application (top), in the presence of 50 µM (DL)-APV (middle), and following APV wash out (bottom). Stimulus artifacts have been omitted for brevity from this and all subsequent electrophysiological traces. B. Cumulative mEPSC amplitude distribution from cell depicted in A before and after APV administration. C. Cumulative inter-event interval distribution before and after APV administration.
Figure 3. Effects of 25 mM ethanol on NMDA mEPSC amplitude and frequency. 
A. Raw data prior to 25 mM ethanol application (top), in the presence of 25 mM ethanol (middle), and following ethanol washout (bottom). B. Cumulative mEPSC amplitude distribution from cell depicted in A before and after ethanol administration. C. Cumulative inter-event interval distribution before and after ethanol administration.

Figure 4. Effects of 50 mM ethanol on NMDA mEPSC amplitude and frequency. 
A. Raw data prior to 50 mM ethanol application (top), in the presence of 50 mM ethanol (middle), and following ethanol washout (bottom). B. Cumulative mEPSC amplitude distribution from cell depicted in A before and after ethanol administration. C. Cumulative inter-event interval distribution before and after ethanol administration.

Figure 5. Effects of 75 mM ethanol on NMDA mEPSC amplitude and frequency. 
A. Raw data prior to 75 mM ethanol application (top), in the presence of 75 mM ethanol (middle), and following ethanol washout (bottom). B. Cumulative mEPSC amplitude distribution from cell depicted in A before and after ethanol administration. C. Cumulative inter-event interval distribution before and after ethanol administration.
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**Figure 6.** Dose-dependent inhibition of asynchronous NMDAR mEPSC amplitude and frequency by 25, 50, and 75 mM ethanol and 50 µM (DL)-APV. **A.** Cumulative reductions in mean amplitude. **= Data attained at 75 mM ethanol differed significantly both from control values (p < 0.03; n=10) and from events recorded in 25 mM ethanol (p < 0.005). * = p < 0.03 relative to control; n=4. ** = p < 0.003; n=10. *** = P < 0.05; n=4. Means comparisons by Student's t-test. Bars = SEM.

**Figure 7.** Ethanol decreases glutamate release probability as indicated by an increase in the paired pulse ratio of NMDAR EPSCs recorded from CA1 pyramidal neurons. **A.** Raw data from a representative cell in the 50 ms inter-stimulus interval group before (left) and after (center) the application of 75 mM ethanol. **Right panel:** Overlay in which the response in ethanol has been scaled to the control response to highlight the change in paired-pulse ratio. **B.** Same as in (A), with a representative cell from the 100 ms inter-stimulus interval group. **C.** Graphical representation of ethanol-induced changes in paired-pulse ratio for all cells from the 50 ms (left; n=6) and 100 ms (right; n=4) inter-stimulus interval groups. **D.** Mean paired-pulse ratio prior to ethanol administration (left) and mean percent facilitation of paired-pulse ratio after 75 mM ethanol application (right) for cells from the 50 vs. 100 ms inter-stimulus interval groups. Ethanol induced statistically significant increases in paired-pulse ratio in both groups (p < 0.05). The difference in the levels of facilitation between the 2 groups was not statistically significant (p = 0.7).
Figure 8. 75 mM ethanol significantly accelerates NMDAR mEPSC decay kinetics. **A.** Left panel: Overlay of averaged mEPSCs from a representative cell before and after application of 75 mM ethanol, displaying amplitude inhibition by ethanol. Right panel: Overlay in which the EPSC in ethanol has been scaled to the peak of the control EPSC to highlight the increased slope of current decay in the presence of ethanol, corresponding with a reduction in decay time constant which was consistent across cells. **B.** Effects of 25, 50, and 75 mM ethanol on decay time constants of NMDAR mEPSCs. * = p < 0.03, n=10. Bars indicate SEM.
Hendricson et al. Fig 1

A

B

Sr²⁺ ACSF

Ca²⁺ ACSF

100 pA

100 mSec

C

Cumulative Probability

Sr²⁺-ACSF

Ca²⁺-ACSF

Inter-event Interval (mS)
Hendricson et al. Fig 5

A

ACS F

75 mM EtOH

Wash

B

Cumulative Probability

NMDA mEPSC Amplitude (pA)

75 mM EtOH

Control

C

Cumulative Probability

Inter-Event Interval (mS)

75 mM EtOH

Control
Hendricson et al. Fig 7

**A**

Control  
75 mM EtOH  
Scaled

**B**

**C**

\[ ISI = 50 \text{ ms} \]

\[ ISI = 100 \text{ ms} \]

**D**

Paired-Pulse Ratio (Control Condition)

<table>
<thead>
<tr>
<th>ISI</th>
<th>50 ms</th>
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<tbody>
<tr>
<td>Ratio</td>
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% Increase in Paired-Pulse Ratio (75 mM EtOH)

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<tr>
<th>ISI</th>
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<tr>
<td>Ratio</td>
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<td>120</td>
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Hendricson et al. Fig 8

A

B

% Mean Decay Time Constant

25 EtOH  50 EtOH  75 EtOH

*