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Inhibition of rat recombinant GluN1/GluN2A and GluN1/GluN2B NMDA receptors by ethanol at concentrations based on the US/UK drink-drive limit

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ABSTRACT

Many studies examine the actions of ethanol on *N*-methyl-D-aspartate (NMDA) receptors using concentrations that are highly toxic (≥100 mM). This study re-assesses the actions of ethanol at concentrations based around the US/UK 'drink-drive' limit (17 mM). Using two-electrode voltage-clamp recordings we examined the actions of ethanol on recombinant GluN1/GluN2A and GluN1/GluN2B NMDA receptors expressed in *Xenopus laevis* oocytes. We also investigated its actions on NMDA receptors containing GluN2A subunits with truncated or deleted carboxy terminal domains. Ethanol inhibition was voltage-independent and for GluN1/GluN2A NMDA receptors mean inhibition (20 mM at −60 mV) was 9.5±0.8% (n=33) while corresponding values for GluN1/GluN2B NMDA receptors were 6.5±0.8% (n=21). EC_{50} values for glutamate at GluN1/GluN2A and glutamate and glycine at GluN1/GluN2B NMDA receptors were unaffected by the presence of ethanol. We did however observe a small increase in glycine potency, in the presence of ethanol, at GluN1/GluN2A NMDA receptors. Neither voltage-dependent Mg^{2+} block nor memantine block was affected by ethanol. Reduced ethanol inhibition was observed however at NMDA receptors containing GluN2A subunits with mutated carboxy terminal domains. We conclude that the levels of inhibition seen with ethanol concentrations near to the US/UK drink-driving limit are very modest and even at higher (intoxicating) concentrations do not alter characteristic NMDA receptor properties.

1. Introduction

N-methyl-D-aspartate (NMDA) receptors are subtypes of the family of ligand-gated ion channels activated by the major excitatory neurotransmitter, L-glutamate. NMDA receptors mediate the slow component of glutamatergic excitatory synaptic currents and the entry of Ca^{2+} into neurones via this receptor channel is the trigger for a cascade of biochemical events that lead to processes such as synaptic plasticity, synaptogenesis and excitotoxicity (Erreger et al., 2004; Chen and Wyllie, 2006). The majority of NMDA receptors are comprised of two GluN1 (NR1) subunits and two GluN2 (NR2) subunits which contain the binding sites for glycine, which acts as a co-agonist at NMDA receptors, and glutamate respectively. The biophysical and pharmacological properties of NMDA receptors are mainly determined by the nature of the GluN2 subunits contained within the receptor assembly. Four GluN2 subunits exist and are termed GluN2A-D which show distinct temporal and spatial expression patterns within the central nervous system. In adult rodent forebrain the majority of NMDA receptors contain either GluN2A or GluN2B NMDA receptor subunits. NMDA receptor function is modulated by a large number of both endogenous and exogenous compounds and of particular interest in this study are the effects of ethanol on GluN2A- and GluN2B-containing NMDA receptors. NMDA receptors are a target for ethanol action and many studies have shown an inhibitory action of this drug at both native and recombinant receptors (Lovinger et al., 1989, 1990; Smothers et al., 2001; Kuner et al., 1993; Masood et al., 1994; Chu et al., 1995; Lovinger, 1995; Mirshahi and Woodward, 1995; Peoples et al., 1997; reviewed in Woodward (1999); Dodd et al. (2000); Allgaier (2002)).

The demonstration that high concentrations of ethanol (≥100 mM) strongly inhibit function is possibly pertinent to chronic ethanol use and alcoholism. Although such ethanol concentrations can be tolerated in chronic alcoholics they are likely to be lethal for many individuals where it is estimated that LD_{50} of ethanol is approximately 85 mM. Clearly during acute social alcohol consumption the levels most individuals experience are much less than 100 mM but are sufficient to affect behaviour. Indeed the current US and UK limit for driving a motor vehicle is 80 mg of alcohol per 100 ml of blood (referred to as a blood alcohol content (BAC) level of 0.08%) – in terms of concentration, this is equivalent to just over 17 mM ethanol.

While regulation of NMDA receptor function is a key determinant of neuronal excitability given the diversity of ion channels that are

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capable of altering neural activity, it is possible that some of the targets of ethanol's action are not directly involved in the process of synaptic transmission *per se* but rather determine intrinsic properties of neurones and their excitability. Thus, in this study we also examined the ability of ethanol to affect the intrinsic excitability of hippocampal neurones as well as NMDA receptor function. Throughout our study we have focussed on assessing ethanol's action at the lower end of the concentration range that is normally used ($\leq$40 mM) and which correspond to 0.6–2.3 times the drink-drive limit. Our data show that the levels of inhibition of both GluN1/GluN2A and GluN1/GluN2B NMDA receptor-mediated currents at ethanol concentrations of 20 mM are very modest (<10%). Furthermore, even at the highest concentration used in this study (80 mM), which would cause severe intoxication and loss of consciousness in many individuals, there are very little effects of ethanol on intrinsic neuronal excitability and the effects of ethanol on NMDA receptor function are not maximal.

2. Materials and methods

2.1. Plasmid constructs, cRNA synthesis and receptor expression in oocytes

The NMDA receptor subunits previously referred to as "NR1", "NR2A" and "NR2B" are now recommended to be called GluN1, GluN2A and GluN2B, respectively (see Alexander et al. (2008); Collingridge et al. (2009)). We adopt this revised nomenclature but when referring to the expression plasmids for the first time below shall use the new and old terminologies for clarity. The pSP64T-derived expression plasmids for rat GluN1-1a (NR1-1a; exon 5 lacking, exons 21 and 22 containing, hereafter referred to as GluN1) and wild-type GluN2A (NR2A) NMDA receptor subunits have been described previously (Chen et al., 2005). The cDNA construct containing the wild-type rat GluN2B (NR2B) subunit (NR2B pBS TR) was a gift from Dr. Stephen Traynelis (Emory University, Atlanta, GA). cRNA was synthesized as runoff transcripts from Mlu I (GluN1 and GluN2A) or Not I (GluN2B) linearized plasmid DNA using either the SP6 (GluN1 and GluN2A) or T7 (GluN2B) polymerase RiboMax RNA synthesis kit (Promega, Madison, WI). Reactions were supplemented with 0.75 mM capping nucleotide, m ^{7}G(5′)ppp(5′)G (Promega, Madison, WI) in the presence of 1.6 mM GTP. Integrity and yield of each synthesized cRNA was verified by fluorescence intensity in ethidium bromide-stained agarose gels. Two GluN2A subunits with altered carboxy terminal domains were also investigated. The GluN2A(trunC) subunit contained residues up to and including Iso1098, while the GluN2A(delC) subunit contained residues up to and including Phe822 (Puddifoot et al., 2009). For recombinant NMDA receptor expression, GluN1 and one of either the GluN2A, GluN2B, GluN2A(trunC) or GluN2A(delC) cRNAs were mixed at a nominal ratio of 1:1 and diluted with nuclease-free water to 5 ng/μl, prior to injection.

Stage V–VI oocytes were obtained from *Xenopus laevis* that had been anaesthetized by immersion in a solution of 3-amino-benzoic acid ethylester (0.5%) and then killed by injection of an overdose solution of pentobarbital (0.4 ml of 20% solution) followed by decapitation and exsanguination after the confirmation of loss of cardiac output. All procedures were carried out in accordance with current UK Home Office regulations. Prior to injection with cRNA mixtures of interest, the follicular membranes of the oocytes were removed. Typically oocytes were injected with between 23–37 nl of cRNA (with the volume injected never exceeding 46 nl). After injection oocytes were placed in separate wells of 24-well plates containing a modified Barth's solution with composition (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgCl_2$ 0.82, $CaCl_2$ 0.77, Tris–Cl 15, adjusted to pH 7.35 with NaOH (Sigma-Aldrich, UK). This solution was supplemented with 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Invitrogen, Paisley, UK). Oocytes were placed in an incubator (19 °C) for 24–48 h to allow for receptor expression and then stored at 4 °C until required for electrophysiological measurements.

2.2. Culture of hippocampal neurones

Primary hippocampal cultures were prepared from E18 Sprague–Dawley rat embryos. Hippocampi were dissected and treated in 0.03% trypsin/EDTA for 15 min at 37 °C, then gently triturated with fire-polished Pasteur pipettes. Viable cells were counted and plated at a density of 55–120 cells mm^{-2} on poly-D-lysine/collagen-coated glass coverslips, then maintained in Neurobasal+B27 supplemented with L-glutamine, penicillin/streptomycin and 1% FBS. 24 h after plating the media was changed and supplemented with fresh media. Cells were fed by replacing half of the media with fresh media on DIV 4, at which point cytosine arabinoside (ara-C; 5 μM) was added to prevent excessive glial cell proliferation.

2.3. Electrophysiological recordings and solutions

Two-electrode voltage clamp recordings were made, using a GeneClamp 500 amplifier (Molecular Devices, Union City, CA), from oocytes that were placed in a solution that contained (in mM): NaCl 115, KCl 2.5, HEPES 10, $BaCl_2$ 1.8, EDTA 0.01; pH 7.3 with NaOH (20 °C). EDTA (10 μM) was added to chelate contaminant extracellular divalent ions, including trace amounts of Zn^{2+}. Current and voltage electrodes were made from thin-walled borosilicate glass (GC150TF-7.5, Harvard Apparatus, Kent, UK) using a PP-830 electrode puller (Narashige Instruments, Japan) and when filled with 3 M KCl and possessed resistances of between 0.5 and 1.5 MΩ. Oocytes were voltage-clamped at −40, −60 or −80 mV. For L-glutamate, concentration–response measurements, the recording solution was further supplemented with glycine (50 μM) and for glycine dose–response measurements this solution was supplemented with glutamate (100 μM). Application of solutions was controlled manually and data were filtered at 10 Hz and digitized at 100 Hz via CED 1401-plus (CED, Cambridge, UK) or Digidata 1200 (Molecular Devices, Union City, CA, USA) A/D interfaces using WinEDR software (Strathclyde Electrophysiology Software, Strathclyde University, UK).

Whole-cell recordings were obtained after 9–10 DIV from pyramidal-like neurones using thick-walled borosillicate glass electrodes with a tip resistance of 5–8 MΩ. The internal solution contained: (in mM) K-gluconate 130, KCl 10, HEPES 10, EGTA 0.1, Glucose 10, Mg-ATP 4, Na-GTP 0.5, Na-phosphocreatine 10, with pH adjusted to 7.3. Experiments were conducted at room temperature in external solution containing: (in mM) NaCl 150, KCl 3, HEPES 10, $MgCl_2$ 2, $CaCl_2$ 3, Glucose 10, (pH 7.3). Current-clamp recordings were performed in the presence of blockers of fast synaptic transmission; 6-cyano-7-nitroquinoxaline 2,3, dione (5 μM), D-2-amino-5-phosphonopentanoic (50 μM) and picrotoxin (50 μM). Access resistances were $\leq$30 MΩ and only cells with stable resting membrane potentials and input resistances were included in the analysis. To measure frequency input (FI) curves, pulses of current (500 ms) in the range +5 – +75 pA were delivered in a random order at 4 s intervals and an average taken over three trials. A −10 pA hyperpolarising pulse lasting 500 ms was included every 30 s to monitor input resistance. Recordings were digitized and analyzed using custom software written in LabView (National Instruments).

For recordings from oocytes, test solutions were applied for 20–60 s or until a plateau to the agonist-evoked response had been achieved. Increasing concentrations of ethanol were applied cumulatively in order to generate concentration–response data in the shortest timeframes possible and to avoid excessively long exposures of oocytes to ethanol or to high levels of agonists which cause 'run-up' in the currents recorded. We confirmed that the inhibitory actions of ethanol applied in this way were readily reversible and levels of inhibition were not different from those obtained when equivalent concentrations were applied separately and oocytes were washed with control solution between applications. All chemicals were purchased from Sigma-Aldrich (Poole, UK) with the exception of the memantine (Tocris Bioscience, Bristol, UK). Ethanol-containing

solutions were derived from 100% ethanol (Sigma) where appropriate volumes were added to achieve final concentrations of 10, 20, 40 or 80 mM.

2.4. Data analysis for dose–response curves

Concentration–response curves were fitted individually for each oocyte with the Hill equation:

$$I = I_{max} / (1 + (EC_{50} / [A])^{n_H})$$

where n_H is the Hill coefficient, I_{max} is the maximum current, [A] is the concentration of agonist, and EC_{50} is the concentration of agonist that produces a half-maximum response. Each data point was then normalized to the predicted fitted maximum of the dose–response curve. The normalized values were then pooled and averaged for each construct and fitted again with the Hill equation, with the maximum and minimum for each curve being constrained to asymptote to 1 and 0 respectively (see Frizelle et al. (2006); Chen et al. (2008); Wyllie and Chen (2007)).

2.5. Statistical analysis

Results are presented as mean ± standard error of the mean and statistical comparison between data sets was assessed using either Student's *t*-test (paired where appropriate) or two-way ANOVA tests (GraphPad Prism v5.0) to determine whether differences were significant ($P<0.05$). Microcal Origin v6.0 software was used for graphical presentation.

3. Results

3.1. Effects of ethanol on intrinsic firing properties of hippocampal neurones

To examine the effects of ethanol on intrinsic firing properties of hippocampal neurones we performed whole-cell patch clamp recordings on cultured hippocampal rat neurons in the presence of blockers of glutamatergic and GABAergic fast synaptic transmission. Neurones were held in current clamp and 500 ms depolarizing current pulses (the range +5 – +150 pA) were applied to initiate action potential firing. Fig. 1A shows recordings (in the absence and presence of ethanol) obtained from an individual neurone in response to a +80 pA current injection. Ethanol (20 or 80 mM) did not affect the resting membrane potential, firing threshold or the current required to initiate firing (Fig. 1B). The mean input resistances of neurones were also not affected by the presence of ethanol being 436 ± 88 MΩ, 435 ± 58 MΩ and 438 ± 68 MΩ for control, 20 and 80 mM ethanol, respectively ($n=7$ cells for each condition) Analysis of frequency input curves recorded under control and ethanol treatments ($n=6$) also indicated that under our recording conditions the mean number of action potentials evoked by the same depolarizing current injection were not significantly different (Fig. 1C). Thus, at the concentrations used in this study modulation of the intrinsic properties of neurones in a way that could impinge on mean activity levels or integration of synaptic input does not appear to be a likely site of action for ethanol. This suggests that targets other than the ion channels responsible for controlling intrinsic firing are the primary site of action of this drug. Of these, NMDA receptors are often considered to be one of the primary mediators of the alterations in excitability induced by ethanol. We describe below the actions of ethanol on the two main subtypes of NMDA receptors with an emphasis on ethanol's actions when applied at concentrations towards the lower end of what are used in many studies.

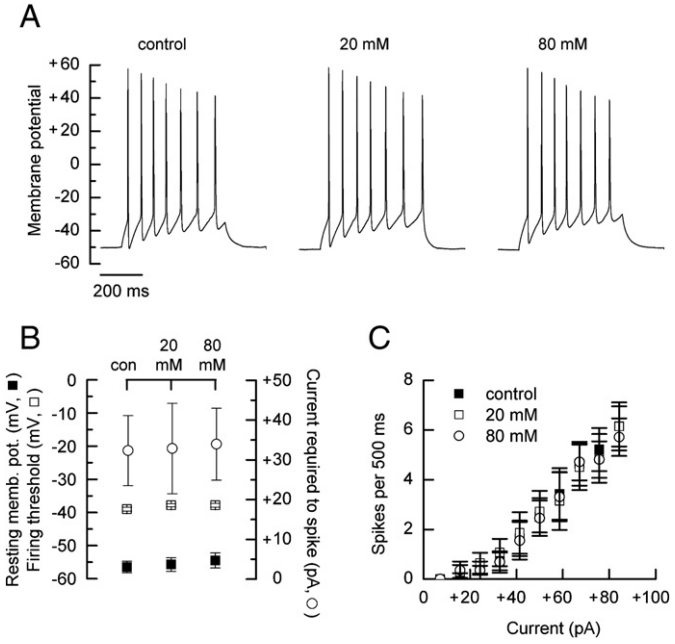

Fig. 1. The effects of ethanol on firing properties of hippocampal pyramidal neurones. (A) Representative current-clamp recordings from a hippocampal pyramidal neurone in response to a depolarizing current injection in the absence and presence of ethanol. (B) Summary of the resting membrane potential, firing threshold and current injection required to initiate an action potential obtained from hippocampal pyramidal neurones ($n=7$ for each parameter). Ethanol (20 or 80 mM) causes no significant differences in the values of these parameters (C) Mean frequency input curves for hippocampal pyramidal neurones obtained in the absence or presence of ethanol ($n=7$). Ethanol (20 or 80 mM) causes no significant differences in the values of these parameters.

3.2. Effect of ethanol on GluN1/GluN2A and GluN1/GluN2B NMDA receptor-mediated responses

Fig. 2A shows that ethanol rapidly and reversibly inhibits NMDA receptor-mediated currents. Even at the highest ethanol concentration used in this study (80 mM), the inhibitory actions of ethanol could be reversed and the magnitude of the glutamate-evoked current returned to its pre-ethanol level within a short time period. Fig. 2B shows that addition of ethanol (10 mM) produces a small inhibition of the NMDA receptor-mediated current and that this level of inhibition is increased when a higher concentration (80 mM) of ethanol is applied. Importantly when the solution is returned to the lower ethanol concentration, the NMDA receptor-mediated current recovers to the level seen when it was applied for the first time. Thus the reversibility and recovery of responses allowed us to apply, cumulatively, different concentrations of ethanol (10–80 mM) to investigate the inhibitory actions of ethanol acting at recombinant GluN1/GluN2A or GluN1/GluN2B NMDA receptors. In a series of control experiments (data not shown) we confirmed that ethanol (10–80 mM) did not affect the holding current required to voltage-clamp un-injected oocytes at potentials of −40, −60 and −80 mV, in either a Ca^{2+}- or Ba^{2+}-containing external recording solution. Fig. 2C shows the mean values of inhibition recorded at −40, −60 and −80 mV for oocytes expressing GluN2A-containing NMDA receptors. A representative two-electrode voltage–clamp current trace recorded at −60 mV in an oocyte expressing GluN1/GluN2A NMDA receptors is shown in Fig. 2D. The inhibition of GluN1/GluN2A NMDA receptor-mediated responses shows no voltage-dependence. For example ethanol (20 mM) displays no significant difference ($P=0.2053$, one-way ANOVA) in the extent of the inhibition of GluN1/GluN2A NMDA receptor-mediated currents) at −40, −60 and −80 mV with mean levels of inhibition being 8.6 ± 1.4% ($n=22$), 9.5 ± 0.8% ($n=33$) and 11.2 ± 0.8% ($n=21$), respectively. While, as illustrated in Fig. 2A, B ethanol inhibition of NMDA receptor-mediated currents is readily

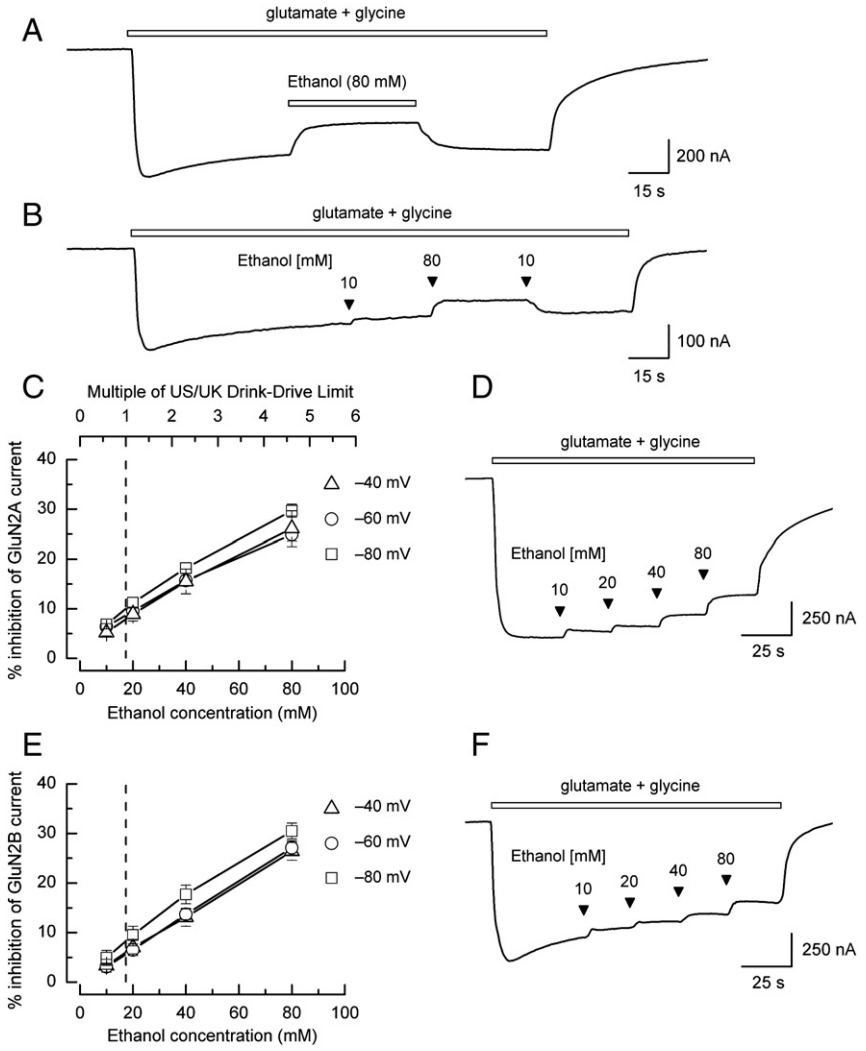

Fig. 2. Ethanol inhibits GluN1/GluN2A and GluN1/GluN2B NMDA receptor-mediated currents in a voltage-independent manner. (A) Two-electrode voltage–clamp current trace from an oocyte at −60 mV to illustrate that inhibition of NMDA receptor-mediated currents by ethanol (80 mM) is readily reversible upon washout. (B) Two-electrode voltage–clamp current trace from an oocyte at −60 mV to illustrate reversibility and recovery of inhibition produced by applying 10 mM, then 80 mM and subsequent return to 10 mM ethanol. (C) Mean percentage inhibition of glutamate-evoked GluN1/GluN2A NMDA receptor-mediated currents produced by ethanol (10–80 mM) at holding potentials of −40, −60 or −80 mV ($n=22$, 33 and 21 respectively). There is no evidence of voltage-dependence to the extent of the block observed. (D) Example two-electrode voltage–clamp current trace recorded from an oocyte at −60 mV and expressing GluN2A-containing NMDA receptors. Application of the glutamate/glycine solution evoked a large inward current whose magnitude was reduced in a step-wise manner as increasing concentrations of ethanol were applied to the external recording solution. (E) Mean percentage inhibition of glutamate-evoked GluN1/GluN2B NMDA receptor-mediated currents produced by ethanol (10–80 mM) at holding potentials of −40, −60 or −80 mV ($n=19$, 21 and 17 respectively). (F) Representative two-electrode voltage–clamp current trace recorded from an oocyte at −60 mV and expressing GluN2B-containing NMDA receptors. Following an initial sag in the response evoked by glutamate/glycine, cumulative application of increasing ethanol concentrations leads to a decrease in the size of the current recorded.

reversible, we also confirmed that inhibiting GluN1/GluN2A NMDA receptor-mediated currents with single applications of ethanol gave similar levels of inhibition (10.4 ± 0.8% at −60 mV, 20 mM ethanol; $n=15$) as were obtained when ethanol was applied at this concentration during cumulative applications. Currents mediated by GluN1/GluN2B NMDA receptors were also inhibited in a voltage independent manner by ethanol with similar levels of inhibition being obtained at this receptor combination as were seen at GluN1/GluN2A NMDA receptors. Fig. 2E shows the mean inhibition values at each of the three holding potentials, while a representative two-electrode voltage–clamp current trace obtained from a GluN1/GluN2B NMDA receptor-expressing oocyte is illustrated in Fig. 2F. For comparison with GluN2A-containing receptors, the mean inhibition of GluN1/GluN2B NMDA receptor-mediated currents at −40, −60 and −80 mV by ethanol (20 mM) is 7.0 ± 1.6% ($n=19$), 6.5 ± 0.8% ($n=21$) and 9.5 ± 1.8% ($n=17$), respectively ($P=0.3043$, one-way ANOVA).

To determine whether ethanol affected the potency of either glutamate or glycine at GluN2A- or GluN2B-containing NMDA receptors we constructed concentration–response curves for each of these agonists in the presence of ethanol. Again we used ethanol at a concentration of 40 mM. Fig. 3A shows a typical two-electrode voltage–clamp current recording from an oocyte expressing GluN1/GluN2A NMDA receptors where cumulative increases in the concentration of glutamate (in the presence of glycine, 50 μM) were applied to determine its EC_{50}, while Fig. 3B shows a two-electrode voltage–clamp current recording obtained by applying increasing concentrations of glycine (in the presence of glutamate, 100 μM) to determine its EC_{50}. The mean concentration–response curves obtained for these agonists are illustrated in Fig. 3C (for GluN1/GluN2A NMDA receptors) and Fig. 3D (for GluN1/GluN2B NMDA receptors). The mean EC_{50} values obtained in the presence of ethanol (40 mM) for glutamate acting at GluN2A-containing NMDA receptors (2.9 ± 0.09 μM, $n_H=1.07\pm0.03$, $n=9$) and glutamate and glycine acting at GluN2B-containing NMDA receptors (1.4 ± 0.1 μM, $n_H=1.09\pm0.04$, $n=8$; 0.74 ± 0.07 μM, $n_H=1.18\pm0.1$, $n=8$, respectively) are not significantly different from the corresponding values for these

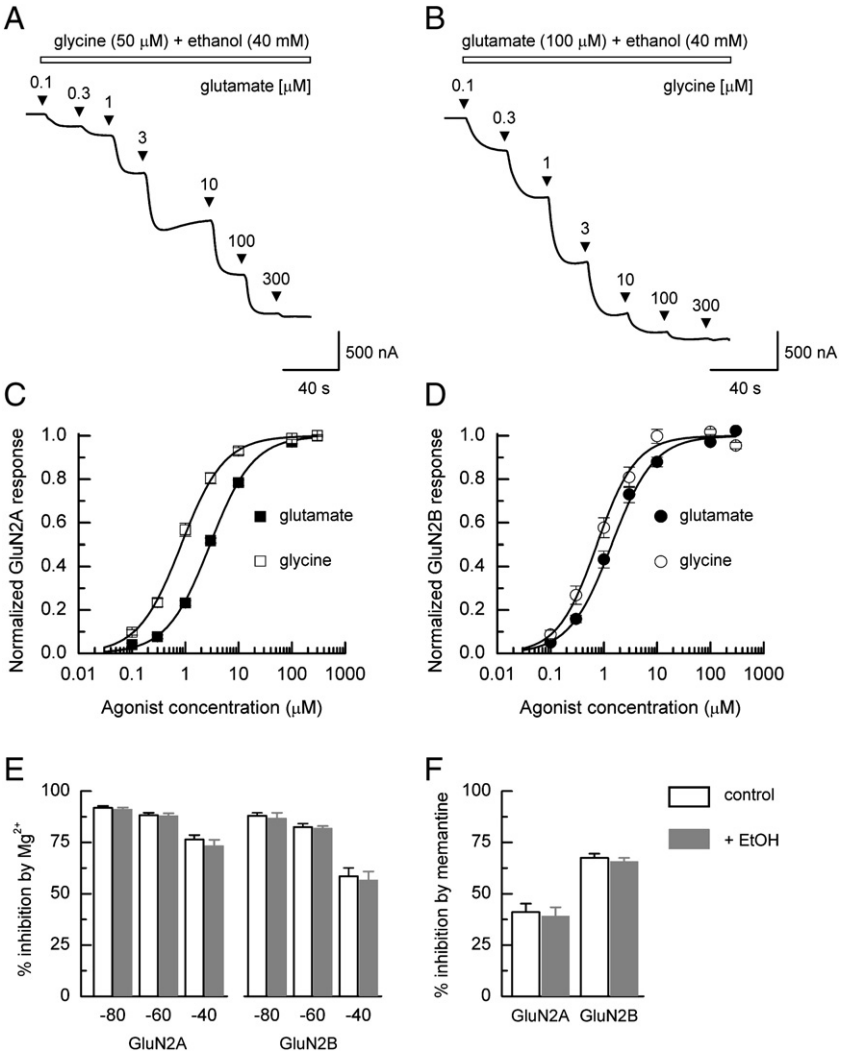

Fig. 3. Glutamate and glycine concentration–response curves and channel-block by Mg^{2+} or memantine are not altered in the presence of ethanol. (A) Example two-electrode voltage–clamp current recording obtained from an oocyte expressing GluN1/GluN2A NMDA receptors. In the presence of glycine (50 μM), increasing concentrations of glutamate (100 nM to 300 μM) were applied cumulatively. (B) as in (A) but showing a two-electrode voltage–clamp current trace obtained in the presence of glutamate (100 μM) and the cumulative addition of increasing concentrations of glycine (100 nM to 300 μM). (C) Mean glutamate (filled squares) and glycine (open squares) concentration–response curves for currents recorded from oocytes expressing GluN1/GluN2A NMDA receptors. The data points are fitted with the Hill equation and give EC_{50} values of 2.9 μM ($n=9$) and 0.84 μM ($n=16$) for glutamate and glycine, respectively. (D) Mean glutamate (filled circles) and glycine (open circles) concentration–response curves for currents recorded from oocytes expressing GluN1/GluN2B NMDA receptors. The data points are fitted with the Hill equation and give EC_{50} values of 1.4 μM ($n=8$) and 0.74 μM ($n=8$) for glutamate and glycine, respectively. (E) Bar graph showing the extent of Mg^{2+}-block (1 mM) of GluN1/GluN2A (black bars; $n=11$) or GluN1/GluN2B (grey bars; $n=11$) NMDA receptor-mediated responses in the absence or presence of ethanol (40 mM). No significant differences are seen in the levels of Mg^{2+}-block at each of the holding potentials examined for either of the NMDA receptor subtypes. (F) Bar graph showing that memantine (3 μM) block of either GluN1/GluN2A ($n=13$) or GluN1/GluN2B ($n=12$) NMDA receptor-mediated currents, recorded at −60 mV, is not affected by the presence of ethanol (40 mM).

receptor subtypes we have reported previously (Erreger et al., 2007; Chen et al., 2008). We did, however, observe an approximately 2-fold increase in glycine potency at GluN2A-containing NMDA receptors in the presence of ethanol (0.84 ± 0.05 μM, $n_H=1.07\pm0.05$, $n=16$) when compared to our previously reported value (Chen et al., 2008) for glycine acting at GluN1/GluN2A NMDA receptors ($P<0.001$, two-tailed *t*-test).

A characteristic feature of NMDA receptors is their voltage-dependent block by a variety of channel blockers and, as such, we investigated whether ethanol affected the extent of block produced by Mg^{2+} and memantine at GluN2A- and GluN2B-containing NMDA receptors. Fig. 3E shows that no significant differences in the amount of block produced by Mg^{2+} (1 mM) at holding potentials of −40, −60 and −80 mV at GluN1/GluN2A or GluN1/GluN2B NMDA receptors were observed when Mg^{2+} was co-applied with ethanol (40 mM). For example, at −60 mV, Mg^{2+} (1 mM) inhibited GluN1/GluN2A NMDA receptor-mediated currents in the absence and presence of ethanol to the same extent ($88\pm1\%$, $n=11$). Similarly, for GluN1/GluN2B NMDA receptor-mediated currents at −60 mV, inhibition by Mg^{2+} in the absence of ethanol was $83\pm2\%$ and in its presence was $82\pm1\%$ ($n=13$). As illustrated in Fig. 3F, the channel-blocking actions of memantine (3 μM) also were not altered in the presence of ethanol (40 mM). Thus, for GluN1/GluN2A NMDA receptors, in the absence of ethanol and at a holding potential of −60 mV, memantine inhibited currents by $41\pm4\%$, whereas in the presence of ethanol the level of inhibition was $39\pm4\%$ ($n=13$). The corresponding values for memantine block of GluN2B-containing NMDA receptors were $68\pm2\%$ and $66\pm2\%$, respectively ($n=12$).

3.3. Effect of ethanol on currents mediated by GluN1/GluN2A NMDA receptors with altered carboxy terminal domains

Several regions in NMDA receptors have been proposed as the target sites at which ethanol exerts its inhibitory actions. These

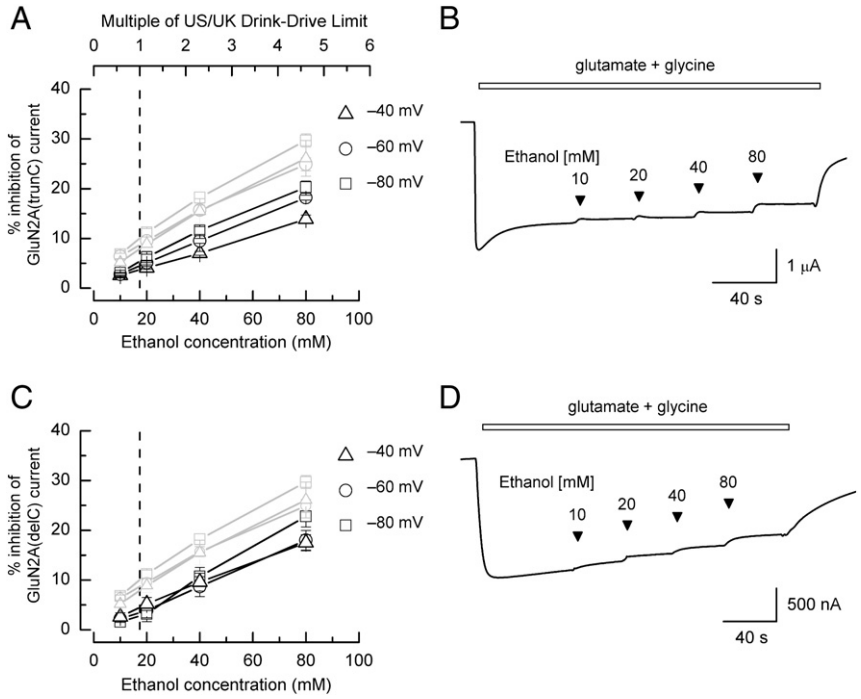

Fig. 4. Reduced ethanol sensitivity of glutamate-evoked currents mediated by NMDA receptors containing GluN2A receptor subunits with truncated or deleted carboxy terminal domains. (A) Mean percentage inhibition of glutamate-evoked GluN1/GluN2A(trunC) NMDA receptor-mediated currents produced by ethanol (10–80 mM) at holding potentials of −40, −60 or −80 mV ($n=8$ for each holding potential). The levels of inhibition observed are less than those seen with wild-type GluN2A-containing NMDA receptors (data shown in grey for comparison). (B) Example two-electrode voltage–clamp current trace recorded from an oocyte at −60 mV and expressing GluN2A(trunC)-containing NMDA receptors. Application of increasing concentrations of ethanol reduces the magnitude of the glutamate/glycine-evoked current. (C) Mean percentage inhibition of glutamate-evoked GluN1/GluN2A(delC) NMDA receptor-mediated currents produced by ethanol (10–80 mM) at holding potentials of −40, −60 or −80 mV ($n=8$, 7 and 8 respectively). Again for comparison the mean data for wild-type GluN1/GluN2A NMDA receptors are shown in grey for comparison. (D) Representative two-electrode voltage–clamp current trace recorded from an oocyte at −60 mV and expressing GluN2A(delC)-containing NMDA receptors; cumulative application of increasing ethanol concentrations leads to a decrease in the size of the current recorded.

include the M3 and M4 regions of the GluN1 subunit (Ronald et al., 2001; Smothers and Woodward, 2006) and the M4 region of the GluN2A subunit (Honse et al., 2004). In addition it has been reported (Peoples and Stewart, 2000) that NMDA receptors containing GluN2B subunits that lack their carboxy terminal domain display a small increase in their sensitivity to ethanol. We have examined whether the ethanol sensitivity of GluN2A-containing NMDA receptors is changed when the carboxy terminal domain of this subunit is either truncated (GluN2A(trunC)) or deleted (GluN2A(delC)). Neither of these constructs showed significant changes in their sensitivity to glutamate (Puddifoot et al., 2009). Fig. 4A shows the mean inhibition produced by ethanol (10–80 mM) acting at GluN1/GluN2A(trunC) NMDA receptors at holding potentials of −40, −60 and −80 mV. A representative two-electrode voltage–clamp current trace recorded at −60 mV is shown in Fig. 4B. Superimposed on the graph in Fig. 4A, and shown in grey, are the data obtained for ethanol inhibition of GluN1/GluN2A NMDA receptor-mediated responses. We observed that ethanol, at all concentrations examined, produced less inhibition at GluN1/GluN2A(trunC) NMDA receptors than was seen at wild-type GluN1/GluN2A NMDA receptors. For example, at −60 mV, ethanol (20 mM) produced significantly less inhibition of GluN1/GluN2A (trunC) NMDA receptor-mediated responses ($5.1\pm0.4\%$, $n=8$), compared to wild-type receptors ($9.5\pm0.8\%$; $P<0.01$). This reduced sensitivity to ethanol was also observed with GluN1/GluN2A(delC) NMDA receptors (Fig. 4C and D). For this receptor combination, application of ethanol (20 mM) at −60 mV, resulted in only $3.9\pm2.0\%$ inhibition ($n=7$).

4. Discussion

Our data confirm ethanol's ability to inhibit NMDA receptor-mediated responses but do not suggest that GluN2A- and GluN2B-containing NMDA receptors show differential sensitivity. While the precise molecular mechanisms by which ethanol inhibits NMDA receptor function remain unclear our data indicate that ethanol's action does not involve altering the potency with which glutamate acts at its binding site nor does it interfere with the potency of the channel blockers Mg^{2+} and memantine. Our results suggest that the carboxy terminal domain of the GluN2A NMDA receptor subunit may play some role in defining the inhibitory actions of ethanol. However, perhaps the main point to make from our study is that ethanol at concentrations which slow an individual's reaction time, causes memory impairment and compromises their ability to drive a motor vehicle safely have very modest effects on NMDA receptor function and no discernible effects on the intrinsic firing properties of hippocampal pyramidal neurones. In regard to this latter point, our data are in agreement with a recent study where ethanol (100 mM) was shown to have no effect on the ability of prefrontal cortex pyramidal neurons to fire action potentials in response to prolonged depolarizing current injections (Tu et al., 2007).

4.1. NMDA receptor subunit dependency of ethanol inhibition

Previous reports have suggested that the extent to which ethanol inhibits NMDA receptors is dependent on their subunit composition with GluN2A- and GluN2B-containing NMDA receptors showing greater inhibition than GluN2C- and GluN2D-containing NMDA receptors (for example see Kuner et al. (1993); Buller et al. (1995); Masood et al. (1994); Chu et al. (1995); Mirshahi and Woodward (1995)). In addition the nature of the GluN1 NMDA receptor subunit contained within the receptor complex has also been reported to influence ethanol sensitivity (Jin and Woodward, 2006). There are conflicting reports regarding whether there is a differential sensitivity of GluN2A- and GluN2B-containing NMDA receptors with some

reports suggesting that ifenprodil sensitive (GluN2B-containing) native NMDA receptors are more sensitive to ethanol than their GluN2A counterparts (Lovinger, 1995, Izumi et al., 2005) while others indicate, for recombinant receptors at least, that they are equally sensitive (Kuner et al., 1993; Masood et al., 1994; Chu et al., 1995; Mirshahi and Woodward, 1995). Our data clearly support the latter conclusion. Furthermore, our data show that the potency of glutamate acting at either GluN2A or GluN2B NMDA receptor subunits was not altered in the presence of ethanol. For GluN1/GluN2B NMDA receptors the potency of the co-agonist, glycine, was not changed, when compared to our previously reported values (Chen et al., 2008) though we did observe an approximately 2-fold increase in glycine potency acting at GluN1/GluN2A NMDA receptors in the presence of ethanol. In contrast, Buller et al. (1995) have reported a small decrease in glycine potency at GluN1/GluN2A NMDA receptors in the presence of higher ethanol concentrations (100 mM) than we have used in the present study. Whether the increase in glycine potency we observe might, in part, counteract some of the inhibitory effects of ethanol would be dependent on the whether the co-agonist binding site on the GluN1 subunit is saturated by the level of glycine normally present in the cerebrospinal fluid. These are thought to be in the micromolar range and at levels that would saturate the GluN1 glycine-binding site, however at synaptic sites the levels of glycine and other putative co-agonists such as D-serine may be regulated by the activity of transporters meaning that lower concentrations exist at such sites (Thomson et al., 1989; Supplisson and Bergman, 1997; Berger et al., 1998; Bergeron et al., 1998). Nevertheless this modest increase in glycine potency at GluN2A-containing NMDA receptors, while indicating that ethanol can exert an action on the glycine-binding GluN1 subunit interaction, if it were to ameliorate ethanol inhibition would most likely do so only when levels of glycine are low. Notwithstanding, none of the results we report here are likely to be influenced by such an effect as we ensured we included saturating levels of glycine (50 μM) in all our recording solutions.

While our study has investigated the actions of ethanol on recombinant NMDA receptors it is of interest to compare our data with the levels of inhibition produced by ethanol acting on synaptically-activated NMDA receptors. The level of inhibition of synaptic NMDA receptors by ethanol (25 mM) has been reported to be around 15–20% (for example see Lovinger et al. (1990); Li et al. (2002); Kash et al. (2008)). However, in such studies, ethanol's actions may not be restricted only to inhibition of NMDA receptors and it may also affect presynaptic function. In this respect it is of interest to note that ethanol inhibition of miniature NMDA receptor-mediated excitatory postsynaptic currents is only apparent when concentrations >50 mM are applied (Hendricson et al., 2004). Thus the levels of ethanol inhibition of recombinant NMDA receptors we report here are comparable with those seen for native NMDA receptors.

4.2. Ethanol and NMDA receptor-channel block

It has been suggested that ethanol interacts with the ability of Mg^{2+} to block NMDA receptors (Martin et al., 1991). Indeed recent work has suggested that for both GluN1/GluN2A and GluN1/GluN2B NMDA receptors an enhanced ethanol inhibition is observed in the presence of Mg^{2+} (Jin et al., 2008). Furthermore, in studies of recombinant NMDA receptors, ethanol shows a reduced ability to block NMDA receptor-mediated currents when these are mediated by receptors that carry point mutations rendering them insensitive to voltage-dependent Mg^{2+} block (Mirshahi and Woodward, 1995). However this latter effect is thought to result from the reduced Ca^{2+} influx that is seen with these mutated receptor channels (Mirshahi et al., 1998). Our study determined whether the extent of Mg^{2+} block was altered in the presence of ethanol. Our results show that for both GluN1/GluN2A and GluN1/GluN2B NMDA receptors that the magnitude of Mg^{2+} (1 mM) block is unaffected by the presence of ethanol at any of the holding potentials investigated. Thus, while the presence of Mg^{2+} may affect the levels of ethanol inhibition seen at these two NMDA receptor subtypes it does not appear that ethanol itself affects the magnitude of the block produced by Mg^{2+}. Since ethanol has been suggested to affect channel blockers of NMDA receptor produced by MK-801 (Spuhler-Phillips et al., 1995) we investigated whether ethanol caused any change in the block produced by memantine. However our experiments showed that when recordings were carried out in the presence of ethanol no change in the extent of the memantine block was evident.

4.3. Reduced ethanol inhibition of responses mediated by GluN1/GluN2A NMDA receptors with altered carboxy terminal domains

We investigated whether levels of ethanol inhibition were affected in receptors that contained GluN2A NMDA receptor subunits in which their carboxy terminal domains had been either truncated or deleted. For both GluN2A(trunC) and GluN2A(delC) subunits we observed significantly less ethanol inhibition compared to that in wild-type GluN2A-containing NMDA receptors. This suggests that, in part, the carboxy terminal domain of this subunit serves some role in mediating the inhibitory actions of ethanol. However, since ethanol inhibition was not completely abolished in the GluN2A(delC) construct the carboxy terminal domain is not solely responsible for mediating the inhibitory effect. Similar types of studies have investigated the role of the carboxy terminal domains of NMDA receptor subunits and ethanol inhibition. Peoples and Stewart (2000) showed that truncation of the GluN1 carboxy terminal domain did not affect the ethanol sensitivity of NMDA receptors containing these and GluN2B subunits whereas truncation of the GluN2B NMDA receptor subunit increased sensitivity to ethanol. In contrast, however a study by Anders et al. (2000) demonstrated that truncation of the GluN1 carboxy terminal domain caused a reduction in the ethanol sensitivity of GluN1/GluN2A but not GluN1/GluN2B NMDA receptors. Moreover, over-expression of a-actinin-2 together with GluN1/GluN2A NMDA receptors caused a reduction in ethanol inhibition of these but not GluN1/GluN2B NMDA receptors (Anders et al., 2000). More recent studies have also suggested a role for actin in the regulation of NMDA receptor sensitivity to ethanol (Offenhäuser et al., 2006; Popp and Dertien, 2008) although a differential effect of ethanol on NMDA receptor-mediated currents in *Eps*8 knockout mice was only apparent when ethanol was applied at a concentration of 400 mM. Intriguingly, the phosphorylation state of the carboxy terminal domain of the GluN2A NMDA receptor subunit has been proposed to influence the sensitivity of GluN1/GluN2A NMDA receptors to ethanol. Anders et al. (1999a) reported that phosphorylation of the carboxy terminal domain of GluN2A, but not GluN2B NMDA receptor subunits by Fyn kinase reduced the level of inhibition produced by ethanol. This effect appears to be specific to phosphorylation by Fyn as in a related study (Anders et al., 1999b), phosphorylation of GluN2A NMDA receptor subunits by Src does not affect their sensitivity to ethanol. Thus, taken together with these earlier studies, our data indicate that in addition to M3 and M4 membrane associated regions (Ronald et al., 2001; Honse et al., 2004; Smothers and Woodward, 2006; Ren et al., 2008) residues within the carboxy terminal domain of the GluN2A NMDA receptor subunit mediate some of the inhibitory actions of ethanol.

4.4. Conclusion

Our study shows that ethanol inhibits recombinant GluN2A- and GluN2B-containing NMDA receptors to a similar extent. This inhibition is not a consequence of a reduced potency of either glutamate or glycine acting at their respective binding sites. Ethanol inhibition does not lead to an alteration of voltage-dependent Mg^{2+} block. For GluN2A-containing NMDA receptors the inhibition, in part, involves

interaction with the intracellular carboxy terminal domain. Finally, the levels of inhibition of NMDA receptor-mediated responses even at ethanol concentrations that would cause severe intoxication in most individuals (80 mM) are comparatively modest (<30%). Nevertheless, when combined with the effects ethanol has on its many other targets in the central nervous system these are likely to contribute to the characteristic physiological and behavioural actions associated with this drug.

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