Effects of Ethanol on GluN1/GluN2A and GluN1/GluN2B NMDA Receptor-Ion Channel Gating Kinetics

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Effects of Ethanol on GluN1/GluN2A and GluN1/GluN2B NMDA Receptor-Ion Channel Gating Kinetics

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Abstract

Background
The N-methyl-D-aspartate receptor (NMDAR) is a major molecular target of alcohol action in the central nervous system, yet many aspects of alcohol’s modulation of the activity of this ion channel remain unclear. We and others have shown that ethanol inhibition of NMDAR involves alterations in gating, especially a reduction in mean open time. However, a full description of ethanol’s effects on NMDAR kinetics, including fitting them to a kinetic model, has not been reported.
Methods
To determine ethanol's effects on NMDAR kinetics, we used steady-state single-channel recording in outside-out patches from HEK-293 cells transfected with recombinant GluN1/GluN2A or GluN1/GluN2B NMDAR subunits. Very low glutamate concentrations were used to isolate individual activations of the receptor.

Results
In both subunit types, ethanol, at approximate whole-cell IC\textsubscript{50} values (156 mM, GluN2A; 150 mM, GluN2B), reduced open probability ($p_o$) by approximately 50% and decreased mean open time without changing the frequency of opening. Open and shut time distributions exhibited two and five components, respectively; ethanol selectively decreased the time constant and relative proportion of the longer open time component. In the GluN2A subunit, ethanol increased the time constants of all but the longest shut time components, whereas in the GluN2B subunit, shut times were unchanged by ethanol. Fitting of bursts of openings (representing individual activations of the receptor) to the gating portion of a kinetic model revealed that ethanol altered two rates: the rate associated with activation of the GluN2A or GluN2B subunit, and the rate associated with the closing of the longer of the two open states.

Conclusions
These results demonstrate that ethanol selectively alters individual kinetic rates and thus appears to selectively affect distinct conformational transitions involved in NMDAR gating.

Graphical Abstract
The N-methyl-D-aspartate receptor (NMDAR) is a major target of alcohol action in the CNS. Ethanol inhibits NMDAR by altering gating of the agonist-bound ion channel. We used singlechannel recording of the two main NMDAR subunits, GluN2A and GluN2B, in the agonistbound state, and found that alcohol affects the rates associated with activation of the GluN2 subunit and closing of the longer of the two open states. Alcohol thus selectively affects distinct conformational transitions involved in NMDAR gating.
INTRODUCTION
Alcohol use disorders present as alterations in behavior, including impairment of motor function, cognition and judgment, and disrupted social interactions during intoxication, but these complex behaviors ultimately arise from changes in synaptic transmission in the brain, due to EtOH-induced alterations in physiological processes including neurotransmitter release, kinase and phosphatase activity, and neuronal ion channel function (Abrahao et al., 2017; McCool, 2011; Ron & Wang, 2009). A large body of evidence supports an important role for the N-methyl-D-aspartate (NMDA) receptor-ion channel in mediating alcohol action in the brain, both in acute intoxication, as well as in phenomena including alcohol craving, tolerance, dependence, withdrawal, and relapse (Chandrasekar, 2013; Holmes et al., 2013; Krystal, Petrakis, Krupitsky, et al., 2003; Krystal, Petrakis, Mason, et al., 2003; Ron & Wang, 2009; Vengeliene et al., 2008). Although alcohol can modulate NMDA receptor sensitivity and signaling via multiple mechanisms (Ron, 2004), its direct action on NMDA receptors involves altering ion channel gating (Lima-Landman & Albuquerque, 1989; Wright et al., 1996) via interaction with specific modulatory sites (Honse et al., 2004; Ren et al., 2003, 2007, 2012; Ronald et al., 2001; Smothers & Woodward, 2006).

Previous studies have shown that EtOH inhibits NMDA receptor gating by decreasing both mean open time and frequency of opening, without appreciably changing closed times (Lima-Landman & Albuquerque, 1989, Wright et al., 1996). These studies established that EtOH inhibition involves modulation of gating but were performed prior to the development of accurate models of ion channel gating, so the precise effects of EtOH on the transitions among the different kinetic states that constitute NMDAR gating behavior have not been determined. In addition, the studies of Lima-Landman and Albuquerque (1989) and Wright et al. (1996) were performed in native CNS neurons in culture. While the conditions used in these studies should closely approximate NMDA receptor modulation by EtOH in vivo, the subunit composition of the receptors tested in these studies is unknown, and most likely was a varying mixture of GluN1/GluN2A, GluN1/GluN2B, and GluN1/GluN2A/GluN2B heterotrimeric NMDA receptors (Al-Hallaq et al., 2007; Rauner & Kohr, 2011). In the present study, we used single-channel recording in recombinant GluN1/GluN2A or GluN1/GluN2B subunit-containing NMDA receptors expressed in the HEK-293 cell line. Using low agonist concentrations to allow the separation of groups of opening events into bursts representing individual activations of the receptor, we fit the data to the gating portion of a cyclic model to determine the kinetic effects of EtOH on NMDA receptor gating. Under these conditions, the effect of EtOH was primarily attributable to the alteration of two specific ion channel gating rate constants in the kinetic model in both subunit types.

MATERIALS AND METHODS
Materials
EtOH (95%, prepared from grain) was obtained from Aaper Alcohol & Chemical Co. (Shelbyville, KY, USA), Cs BAPTA was obtained from Invitrogen ThermoFisher (Waltham, MA), and all other drugs and chemicals were obtained from MilliporeSigma (St. Louis, MO, USA).
Cell culture and transfection

Human embryonic kidney (HEK) 293 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured as previously described (Ren et al., 2017). Cells were allowed to grow to 75–90% confluence before transient transfection with plasmids containing rat GluN1-1a (pRC) and GluN2A (pcDNA1) or GluN2B (pDP3) subunits and green fluorescent protein (Addgene) at a ratio of 2:2:1 using a calcium phosphate transfection kit or Lipofectamine 3000 (Invitrogen ThermoFisher). During and after the transfection, 200 μM D,L-2-amino-5-phosphonovaleric acid (APV) and either 100 μM ketamine or 100 μM memantine were added to the culture media to protect cells from glutamate excitotoxicity. Cells were used in experiments 18–72 h after transfection.

Electrophysiological recording

Patch-clamp recording was performed at room temperature using an Axopatch 1D or Axopatch 200B (Molecular Devices) amplifier. Patch-pipets were coated with R6101 elastomer (Dow-Corning) and had tip resistances of 8–15 MΩ following heat polishing. Outside-out patches were voltage-clamped at −50 mV and superfused in Mg²⁺-free external recording solution containing (in mM) 150 NaCl, 5 KCl, 0.2 CaCl₂, 10 HEPES, 10 μM EDTA, 10 glucose, and 10 sucrose. Solution pH was adjusted to 7.4 by adding a calculated ratio of HEPES free acid to HEPES sodium salt (Buffer Calculator, available at https://www.liverpool.ac.uk/pfg/Tools/BuffferCalc/Buffer.html). Ultrapure salts and chemicals were used to minimize contamination by other cations. The intracellular recording solution contained (in mM) 140 CsCl, 2 Mg₄ ATP, 10 BAPTA, and 10 HEPES; pH was adjusted to 7.2 with CsOH. Solutions of agonists and EtOH were prepared fresh daily in extracellular solution and applied to cells using a stepper motor-driven rapid solution exchange apparatus (Fast-step, Warner Instrument Co.) and 600 μm id square glass tubing. The EtOH concentrations used were 156 mM (GluN2A) and 150 mM (GluN2B), which we have found in preliminary experiments and previous results using whole-cell patch-clamp recording to be the approximate IC₅₀ values; we have previously reported similar values (Ren et al., 2003, 2012; Zhao et al., 2015), although other laboratories have reported greater differences in sensitivity between the GluN2A and GluN2B subunits (Smothers et al., 2001). Recordings alternated between control and EtOH exposure at one- or two-minute intervals in order to minimize the influence of any slow changes in ion channel activity. Low concentrations of glutamate in the presence of a saturating concentration of glycine were used to obtain widely separated individual receptor activations (Gibb & Colquhoun, 1991, 1992; Wyllie et al., 1998). Each patch was exposed to an initial concentration of 100 nM glutamate; in some cases, a concentration of 1 μM glutamate was used in patches exhibiting very low levels of activity.

Data analysis

Data from single-channel recordings were acquired at 50 kHz, digitally filtered at 5 kHz (8-pole Bessel), and idealized using the segmentation K-means algorithm in the QUB software suite (Qin, 2004). Open and shut dwell time histograms were fitted with multiple exponential components using Channelab (Synaptosoft) after imposing a 50–100 μs dead time, and mean open times were obtained from the proportionally-weighted averages of the individual components. The frequency of opening was calculated by dividing the number of openings by the recording time. Data were obtained from seven patches for each subunit combination. Patches used for each subunit combination had one to three levels of opening and were obtained on multiple experimental days over the course of several weeks.
Burst analysis was performed in steady-state single-channel records by using values of the critical time interval \( \tau_{\text{crit}} \) from shut time histograms that minimized the total number of misclassified events. Bursts with more than one level of opening were excluded from the analysis. The values for \( \tau_{\text{crit}} \) were calculated to be between the third and fourth components of the shut time in GluN2A and between the fourth and fifth components of the shut time in GluN2B in order to isolate burst events corresponding to individual receptor activations.

Kinetic modeling
Opening and closing events within bursts were fitted to a kinetic model using the maximum interval likelihood (MIL) function of the QUB program in order to obtain values for the rate constants for subunit activation and channel opening (Qin & Li, 2004). All parameters were allowed to vary freely. We chose to use a simple cyclic gating model of Traynelis and colleagues (Erreger, Geballe, et al., 2005), which is able to fit both single-channel and macroscopic response data for the GluN2A, GluN2B, and GluN2C subunits (Banke & Traynelis, 2003; Erreger et al., 2007; Erreger, Dravid, et al., 2005; Erreger, Geballe, et al., 2005), and which has kinetic states that appear to correspond to the main conformational states of the receptor-ion channel protein (Banke & Traynelis, 2003).

Statistical analysis
Effects of EtOH on single-channel kinetic measures and rate constants obtained from model fitting were compared in the same patches using paired \( t \)-tests or repeated-measures ANOVA.

RESULTS
Effect of EtOH on NMDA receptor single-channel conductance
In outside-out patches from cells expressing GluN1/GluN2A or GluN1/GluN2B NMDA receptor subunits, low concentrations of glutamate (0.1–1 μM) in the presence of a saturating concentration of glycine (50 μM) elicited a low level of NMDA receptor activity, with groups of openings widely separated by long closings (Figure 1). This pattern of activity was observed in both the absence and the presence of EtOH (156 mM, GluN2A; 150 mM, GluN2B). EtOH did not alter the single-channel chord conductance determined from all-points histograms (Figure 2; GluN2A: 54.6 ± 1.63 vs. 56.7 ± 2.59 pS in the absence and presence of EtOH, respectively; paired \( t \)-test, \( p > 0.05 \), \( N = 7 \) patches; GluN2B: 54.2 ± 1.23 vs. 52.5 ± 1.75 pS in the absence and presence of EtOH, respectively; paired \( t \)-test, \( p > 0.05 \), \( N = 7 \) patches), and did not produce any evident alteration in the appearance of the single-channel records, such as flickering block (Figures 3A,C and 4A,C).
FIGURE 1. Single-channel currents activated by 0.1 μM glutamate and 50 μM glycine in outside-out patches from cells expressing GluN1/GluN2A (A, B) or GluN1/GluN2B (C, D) NMDA receptor subunits in the absence (A, C) and the presence (B, D) of EtOH, 156 mM (B) or 150 mM (D). Channel openings are downward. Scale bars in A apply to all records.

FIGURE 2. Open-point histograms of glutamate-activated single-channel current amplitudes in outside-out patches from cells expressing GluN1/GluN2A (A) or GluN1/GluN2B (B) subunits in the absence (black) and the
presence (gray) of EtOH, 156 mM (A) or 150 mM (B). Curves shown are least-squares fits of a Gaussian function to the data in the absence (solid blue) and presence (dashed red) of EtOH.

FIGURE 3. (A, C) Single-channel currents activated by 0.1 μM glutamate and 50 μM glycine in an outside-out patch from a cell expressing GluN1/GluN2A subunits in the absence (A) and the presence (C) of 156 mM EtOH. Patches were alternately exposed to control and EtOH solutions at 2-min intervals. Channel openings are downward. (B, D) Open time histograms of data from a typical outside-out patch in the absence (B) and presence (D) of EtOH, 156 mM. Curves shown are the maximum likelihood of multiple exponential fits to the data. Histograms were well fitted by two components. Arrows denote the peaks of the longer components; the dotted line in D indicates the position of the peak in the absence of EtOH.

FIGURE 4. (A, C) Single-channel currents activated by 0.1 μM glutamate and 50 μM glycine in an outside-out patch from a cell expressing GluN1/GluN2B subunits in the absence (A) and the presence (C) of 150 mM EtOH. Patches were alternately exposed to control and EtOH solutions at 1-min intervals. Channel openings are downward. (B, D) Open time histograms of data from a typical outside-out patch in the absence (B) and presence (D) of EtOH, 150 mM. Curves shown are the maximum likelihood of multiple exponential fits to the data. Histograms were well fitted by two components. Arrows denote the peaks of the longer components; the dotted line in D indicates the position of the peak in the absence of EtOH.

Effects of EtOH on open times

Open time distributions for glutamate-activated current in GluN1/GluN2A or GluN1/GluN2B NMDA receptors could be adequately fitted by two components (GluN2A: 62.6 ± 6.24 μs and 4.01 ± 0.441 ms;
GluN2B: 130 ± 16.9 μs and 5.39 ± 0.234 ms) with similar proportions of openings (GluN2A: 48.3 ± 2.38% and 51.8 ± 2.39%, and GluN2B: 49.7 ± 3.81% and 50.3 ± 3.81% for the fast and slow components, respectively, Figures 3B and 4B). The mean open times were 3.21 ± 0.341 ms (GluN2A) and 3.51 ± 0.216 ms (GluN2B). In the presence of EtOH mean open time was decreased in GluN2A by 33% to 2.17 ± 0.243 ms (Figure 3D; p < 0.001, paired t-test, N = 7 patches), and in GluN2B by 42% to 2.05 ± 0.376 ms (Figure 4D; p < 0.01, paired t-test, N = 7 patches). Open time distributions in the presence of EtOH were also well fitted by two components; the time constant of the fast component was not significantly changed compared with the control condition (GluN2A: 50.3 ± 3.97 μs; paired t-test, p > 0.05, N = 7 patches; GluN2B: 138 ± 42.1 μs; paired t-test, p > 0.05, N = 7 patches), but that of the slow component was significantly decreased (GluN2A: 3.07 ± 0.345 ms; paired t-test, p < 0.001, N = 7 patches; GluN2B: 3.59 ± 0.269 ms; paired t-test, p < 0.01, N = 7 patches). In the GluN2A subunit, but not the GluN2B subunit, the areas of both components were significantly changed, such that there were relatively more fast openings in the presence of EtOH (62.0 ± 4.11% and 38.0 ± 4.12% for the fast and slow components, respectively; paired t-test, p < 0.05, N = 7 patches). By contrast, the overall frequency of opening was not changed by EtOH in either subunit type (GluN2A: 7.91 ± 1.96 vs. 7.34 ± 1.30 for control and EtOH-treated patches, respectively; paired t-test, p > 0.05, N = 7 patches; GluN2B: 15.8 ± 6.52 vs. 13.6 ± 5.99 for control and EtOH-treated patches, respectively; paired t-test, p > 0.05, N = 7 patches).

Effects of EtOH on shut times
For both subunit types, shut time distributions for patches in the absence of EtOH could be fitted with five exponential components ranging from approximately 50 μs to 400 ms (Table 1 and Figure 4). Similar distributions were also obtained in the same patches exposed to EtOH. In contrast to its effects on open time, EtOH did not alter any of the shut time constants (repeated-measures ANOVA, p > 0.05, N = 7 patches) or their relative areas (repeated-measures ANOVA, p > 0.05, N = 7 patches). As would be expected from the observation above that EtOH did not produce a flickering block, EtOH did not introduce any additional shut times.

**TABLE 1. Effects of EtOH on shut times**

<table>
<thead>
<tr>
<th></th>
<th>(\tau_1 (\mu s))</th>
<th>(\tau_2)</th>
<th>(\tau_3)</th>
<th>(\tau_4)</th>
<th>(\tau_5)</th>
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</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>47.6 ± 2.24</td>
<td>0.267 ± 0.0125</td>
<td>3.02 ± 0.382</td>
<td>52.2 ± 12.2</td>
<td>419 ± 37.3</td>
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<td></td>
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<td>(19.9)</td>
<td>(16.3)</td>
<td>(10.0)</td>
<td>(15.1)</td>
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<tr>
<td>EtOH</td>
<td>51.3 ± 3.87</td>
<td>0.319 ± 0.0284</td>
<td>2.74 ± 0.272</td>
<td>41.7 ± 11.3</td>
<td>395 ± 46.1</td>
</tr>
<tr>
<td></td>
<td>(40.0)</td>
<td>(16.0)</td>
<td>(15.2)</td>
<td>(7.5)</td>
<td>(18.2)</td>
</tr>
<tr>
<td><strong>GluN2B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>51.0 ± 11.8</td>
<td>0.435 ± 0.0712</td>
<td>7.08 ± 2.22</td>
<td>24.1 ± 3.03</td>
<td>262 ± 56.6</td>
</tr>
<tr>
<td></td>
<td>(56.0)</td>
<td>(14.5)</td>
<td>(10.0)</td>
<td>(13.6)</td>
<td>(6.4)</td>
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<tr>
<td>EtOH</td>
<td>54.0 ± 15.4</td>
<td>0.424 ± 0.0846</td>
<td>10.4 ± 2.62</td>
<td>53.7 ± 22.5</td>
<td>524 ± 84.3</td>
</tr>
<tr>
<td></td>
<td>(51.5)</td>
<td>(16.7)</td>
<td>(10.9)</td>
<td>(11.9)</td>
<td>(9.3)</td>
</tr>
</tbody>
</table>

**Note:** GluN1/GluN2A and GluN1/GluN2B NMDA receptor shut times from multiple exponential fits to shut time histograms from outside-out patches under control conditions and in the presence of EtOH, 156 mM (GluN2A) or 150 mM (GluN2B). Time constant (t) values are in ms, except where indicated, and are expressed as the mean ± SE obtained from N = 7 patches. Values in parentheses are the percentage of the total shut time for each component.
Fitting of burst data to a kinetic model

A distinct separation was evident in the shut time distributions between the third and fourth components in GluN2A subunit-containing receptors and between the fourth and fifth components in GluN2B subunit-containing receptors. In the GluN2A subunit, the shortest three shut times are considered to occur within an individual activation of the receptor-ion channel (Erreger, Dravid, et al., 2005; Gibb & Colquhoun, 1991). Of the longest two shut times, one represents a long-lived desensitized state (within an activation), and one is the agonist-unbound state (between activations). Although there is evidence from some studies that EtOH may influence NMDAR desensitization, the absence of a change in the closed time corresponding to the desensitized state in the presence of EtOH is consistent with a negligible effect under the conditions used in this study. In the GluN2B subunit, the longest two shut times are considered to differentiate individual activations of the receptor-ion channel (Erreger, Dravid, et al., 2005). To determine the effects of EtOH on gating transitions in the fully-ligated, non-desensitized receptor, we grouped opening events into bursts that should correspond to individual activations of the receptor by choosing values of $\tau_{\text{crit}}$ between the third and fourth (GluN2A) or fourth and fifth (GluN2B) shut time components (Figure 5, arrows). Values of $\tau_{\text{crit}}$ determined for patches in the absence and presence of EtOH were 10.7 and 8.9 ms, respectively for GluN2A and 83.3 and 151 ms, respectively for GluN2B. We extracted bursts from the idealized data record such that closed times longer than the $\tau_{\text{crit}}$ value were not included in the analysis. Maximal interval likelihood fitting of bursts to scheme 1 of Erreger, Geballe, et al. (2005), a cyclic model that does not incorporate agonist binding and unbinding steps or desensitized states (Figure 6), yielded values for the rate constants that in general were similar to those previously determined (Table 2; Erreger, Geballe, et al., 2005). In both GluN2A and GluN2B subunits, EtOH selectively altered two of the kinetic transitions: It decreased the rate constant for the slow forward rate, $k_{s+}$, by 28.4% (paired $t$-test, $p < 0.0005$) in GluN2A and by 30.2% (paired $t$-test, $p < 0.001$) in GluN2B, and increased the reverse rate between open states, $o_-$, by 49.8% ($t$-test, $p < 0.001$) in GluN2A and 31% ($t$-test, $p < 0.05$) in GluN2B. The remaining kinetic values were not appreciably altered by EtOH (mean change: 8.0 percent; $t$-tests, $p > 0.05$). To test whether the two changed rate constants could account for the observed decreases in mean open time and open probability due to EtOH, we substituted these rates in the model and generated simulated current traces using the QUB program, leaving all other rates unchanged from their control values. Changing the slow forward rate, $k_{s+}$, and the open state reverse rate, $o_-$, to their values in the presence of EtOH accounted for 91% and 90% of the inhibitory effect of EtOH on open probability in the GluN2A and GluN2B subunits, respectively.
FIGURE 5. Shut time histograms of data from individual outside-out patches from cells expressing GluN1/GluN2A (A, B) or GluN1/GluN2B (C, D) subunits in the absence (A, C) and presence (B, D) of EtOH. Curves shown are maximum likelihood multiple exponential fits; five components were required to adequately fit the data. Arrows indicate the critical time interval \( t_{\text{crit}} \) for each shut time histogram calculated to minimize the total number of misclassified events.

FIGURE 6. Fitting individual NMDA receptor activations (bursts) to a cyclic kinetic model. (A, B) Single-channel currents activated by 0.1 \( \mu \text{M} \) glutamate and 50 \( \mu \text{M} \) glycine in an outside-out patch from a cell expressing GluN1/GluN2A subunits in the absence (A) and the presence (B) of 156 mM EtOH. (C, D) Single-channel currents activated by 0.1 \( \mu \text{M} \) glutamate and 50 \( \mu \text{M} \) glycine in an outside-out patch from a cell expressing GluN1/GluN2B subunits in the absence (C) and the presence (D) of 150 mM EtOH. Channel openings are downward. Blue shading indicates groups of openings ("bursts") defined by a critical time interval and considered to represent individual receptor activations. (E) A cyclic kinetic model of the gating of fully-bound GluN1/GluN2A NMDA receptors (Erreger, Geballe, et al., 2005). Opening and closing events within bursts were fitted to the model using the maximum interval likelihood (MIL) function of the QUB program (Qin & Li, 2004) in order to obtain values for the rate constants for subunit activation and channel opening. Kinetic rates altered by EtOH are
indicated by red dashed circles. Abbreviations: A, agonist; R, receptor; O, open; f, fast; s, slow; +, forward; −, reverse.

### TABLE 2. Effects of EtOH on gating rate constants

<table>
<thead>
<tr>
<th></th>
<th>$k_{f+}$</th>
<th>$k_{f-}$</th>
<th>$k_{s+}$</th>
<th>$k_{s-}$</th>
<th>$o_{+}$</th>
<th>$o_{-}$</th>
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<tr>
<td>GluN2A</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>4110 ± 207</td>
<td>2860 ± 379</td>
<td>423 ± 21.3</td>
<td>956 ± 126</td>
<td>4880 ± 407</td>
<td>657 ± 67.8</td>
</tr>
<tr>
<td>EtOH</td>
<td>3870 ± 272</td>
<td>2600 ± 275</td>
<td>303 ± 14.3***</td>
<td>829 ± 88.3</td>
<td>5063 ± 498</td>
<td>984 ± 104***</td>
</tr>
<tr>
<td>GluN2B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3540 ± 436</td>
<td>2120 ± 310</td>
<td>56 ± 3.1</td>
<td>1040 ± 98.5</td>
<td>3020 ± 361</td>
<td>379 ± 14.2</td>
</tr>
<tr>
<td>EtOH</td>
<td>3200 ± 495</td>
<td>2830 ± 870</td>
<td>39 ± 3.01***</td>
<td>993 ± 172</td>
<td>2540 ± 512</td>
<td>497 ± 40.6*</td>
</tr>
</tbody>
</table>

*Note:* Rate constants obtained from fitting open and shut events within bursts to a kinetic model (Figure 5C). Values (in s$^{-1}$) are the mean ± SE from $N$ = 7 patches. Asterisks denote significant differences from the control (paired t-tests; ***$p < 0.001$, *$p < 0.05$).

### DISCUSSION

In the present study, we used recombinant rat GluN1/GluN2A and GluN1/GluN2B NMDAR to demonstrate that inhibition by EtOH at its approximate IC$_{50}$ values (GluN2A: 156 mM; GluN2B: 150 mM in this study) involved a decrease in mean open time without appreciable changes in the frequency of opening, single-channel conductance, or shut time distribution, and that the effect of EtOH was attributable to selective modulation of two specific ion channel gating rate constants in the kinetic model used.

Following the initial demonstrations that EtOH inhibits NMDA receptor activity (Hoffman et al., 1989; Lima-Landman & Albuquerque, 1989; Lovinger et al., 1989), a few studies used the single-channel recording to examine the alterations in receptor kinetics responsible for the inhibitory effect. In the earliest such study, Lima-Landman and Albuquerque (1989) reported that EtOH (86.5 and 174 mM) decreased NMDAR mean open time in outside-out patches from rat hippocampal pyramidal neurons without altering single-channel conductance. These investigators observed a decrease in open probability of approximately 50% but did not report values for opening frequency. Wright et al. (1996) subsequently showed that in outside-out patches from mouse cerebral cortical neurons, EtOH (200 mM) decreased both mean open time and frequency of opening without altering single-channel conductance or shut times, and without introducing fast flickering behavior. The reduction in mean open time was greater than that in the frequency of opening, but both were required to account for the observed reduction in whole-cell current by EtOH. In the present study, at a concentration similar to those used previously, but in recombinant rat GluN1/GluN2A or GluN1/GluN2B NMDA receptors expressed in a cultured cell line rather than in native CNS neurons, we observed similar effects of EtOH: The main effect was to decrease mean open time of the ion channel without altering single-channel conductance. There was also no difference in closed-time distributions, including no introduction of very short closed times, or fast flickering behavior. Because either an increase in very short closed times or reduced single-channel conductance could indicate an open-channel block mechanism (Hille, 2001), the absence of either finding has been interpreted as evidence that EtOH does not act by occluding the ion channel (Wright et al., 1996). The observation that EtOH primarily shortens mean open time is consistent with a mechanism of inhibition involving an increase in the rate of ion channel closing.
Previous studies have reported that magnesium ions, which normally block NMDA receptors at strongly negative potentials under physiological conditions, can enhance EtOH inhibition of NMDA receptors (Chandler et al., 1994; Chu et al., 1995; Martin et al., 1991; Michaelis & Michaelis, 1994; Morrisett et al., 1991), although this has not been observed in all studies (Bhave et al., 1996; Chu et al., 1995; Peoples et al., 1997). In the present study, experiments were performed in the absence of extracellular magnesium to avoid block at negative membrane potentials. It is thus possible that this resulted in a lesser degree of EtOH inhibition than would be observed at physiological concentrations of magnesium. In addition, because magnesium alters gating kinetics (Kampa et al., 2004), it is also possible that magnesium, if it had been present, could have altered the effects of EtOH on the kinetic model. Many early studies also demonstrated glycine modulation of EtOH inhibition of NMDA receptors and proposed glycine antagonism as a mechanism of inhibition (Buller et al., 1995; Dildy-Mayfield & Leslie, 1991; Rabe & Tabakoff, 1990; Woodward & Gonzales, 1990); however, the binding of glycine or glutamate is not altered by EtOH (Snell et al., 1993), nor is EtOH inhibition competitive with respect to glycine or glutamate (Cebers et al., 1996; Gonzales & Woodward, 1990; Peoples et al., 1997; Peoples & Weight, 1992; Woodward, 1994). In the present study, we therefore excluded transitions involved in agonist or coagonist binding by fitting events occurring during bursts, which represent single activations of the ion channel (Gibb & Colquhoun, 1991, 1992), and are separated by one or two long shut times attributable to agonist dissociation and (in the GluN2A subunit) desensitization. Thus we attempted to model the effect of EtOH on the gating of the fully-ligated, non-desensitized receptor. Fitting of events during bursts in the absence and presence of EtOH to a simple cyclic model using the maximum interval likelihood (MIL) function of the QUB program revealed that two rate constants in the gating section of the model were appreciably affected by EtOH in both GluN1/GluN2A and GluN1/GluN2B receptors: The slow forward rate, $k_{s+}$, in the cyclic portion of the model was decreased, and the main open state reverse rate, $o_-$, was increased. The slow forward rate, along with the slow reverse rate, $k_{s-}$, has been attributed to conformational changes in the GluN2 subunit (Banke & Traynelis, 2003). Thus this result is consistent with EtOH acting via the GluN2 subunit, and the lack of effect of EtOH on the fast forward rate indicates that the corresponding transition in the GluN1 subunit is insensitive to EtOH, which could be due to structural differences between the subunit types underlying the much faster transitions in the GluN1 subunit. The main open state reverse rate affects the longer of the two open times, such that increasing this rate decreases the duration of the longer component. The longer open state accounts for the majority of charge transfer mediated by the NMDAR, so preferentially shortening it has a relative effect greater than would be caused by a similar change to the shorter component. Our simulations indicated that the two states that were measurably altered by EtOH accounted for the great majority of its inhibitory effect.

Although EtOH can modulate the activity of NMDA receptors via multiple actions, such as by altering phosphorylation and interacting proteins (Ron, 2004), in this study, we have chosen to focus on its direct action on the NMDA receptor channel, which can be observed under conditions in which intracellular mechanisms of modulation are minimized or eliminated (Lovinger et al., 1989; Peoples et al., 1997), and which occurs at intoxicating concentrations (Hoffman et al., 1989; Lima-Landman & Albuquerque, 1989; Lovinger et al., 1989). A consideration in the present study is that interacting mechanisms present in CNS neurons may be absent in the expression system used. It should also be noted that the concentrations used in the present study are above the normal range for intoxication in
humans (~20–50 mM), although even greater concentrations than those used here may be reached in highly-tolerant individuals (Abrahao et al., 2017). Concentrations at approximate IC_{50} values were used in the present study to ensure that changes in individual microscopic kinetic rates, which act in concert to determine NMDA receptor gating, would be detectable. Results of many studies from this and other laboratories showing sigmoidal EtOH concentration-response curves with slopes of approximate unity are consistent with the view that lower concentrations of EtOH would have qualitatively similar effects (Honse et al., 2004; Jin & Woodward, 2006; Peoples et al., 1997; Ren et al., 2012; Smothers & Woodward, 2006; Zhao et al., 2015). Available evidence supports the view that the NMDA receptor is responsible for some of the effects of intoxicating EtOH concentrations in humans (Holmes et al., 2013; Jury et al., 2018; Krishnan-Sarin et al., 2015; Krystal, Petrakis, Krupitsky, et al., 2003; Radke et al., 2017). We and others have identified clusters of adjacent residues in the third and fourth membrane-associated domains of the GluN1 (Jin & Woodward, 2006; Ren et al., 2012; Ronald et al., 2001; Smothers & Woodward, 2006) and GluN2 (Honse et al., 2004; Ren et al., 2003, 2007, 2008, 2012; Wu et al., 2019; Zhao et al., 2015) subunits of the NMDA receptor that strongly regulate EtOH sensitivity, and which appear to form the sites by which EtOH inhibits the receptor under the conditions used in this study. While significant advances have been made in understanding the conformational changes that underlie ion channel gating (Glasgow et al., 2015; Hansen et al., 2021; Karakas & Furukawa, 2014; Kazi et al., 2013; Lee et al., 2014), and the available evidence is consistent with movements of the alcohol-sensitive side chains relative to each other during gating transitions, further studies will be required to determine how the EtOH molecule interacts with these domains to alter the kinetics of the conformational changes that are responsible for the rate constants that are regulated by EtOH.

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CONFLICT OF INTEREST
The authors have no conflicts of interest to disclose.

References


