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Interactions among Positions in the Third and Fourth Membrane-associated Domains at the Intersubunit Interface of the *N*-Methyl-D-aspartate Receptor Forming Sites of Alcohol Action

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Abstract: The *N*-methyl-D-aspartate (NMDA) glutamate receptor is a major target of ethanol in the brain. Previous studies have identified positions in the third and fourth membrane-associated (M) domains of the NMDA receptor GluN1 and GluN2A subunits that influence alcohol sensitivity. The predicted structure of the NMDA receptor, based on that of the related GluA2 subunit, indicates a close apposition of the alcohol-sensitive positions in M3 and M4 between the two subunit types. We tested the hypothesis that these positions interact to regulate receptor kinetics and ethanol sensitivity by using dual substitution mutants. In single-substitution mutants, we found that a position in both subunits adjacent to one previously identified, GluN1(Gly-638) and GluN2A(Phe-636), can strongly regulate ethanol sensitivity. Significant interactions affecting ethanol inhibition and receptor deactivation were observed at four pairs of positions in GluN1/GluN2A: Gly-638/Met-823, Phe-639/Leu-824, Met-818/Phe-636, and Leu-819/Phe-637; the latter pair also interacted with respect to desensitization. Two interactions involved a position in M4 of both subunits, GluN1(Met-818) and GluN2A(Leu-824), that does not by itself alter ethanol sensitivity, whereas a previously identified ethanol-sensitive position, GluN2A(Ala-825), did not unequivocally interact with any other position tested. These results also indicate a shift by one position of the predicted alignment of the GluN1 M4 domain. These findings have allowed for the refinement of the NMDA receptor M domain structure, demonstrate that this region can influence apparent agonist affinity, and support the existence of four sites of alcohol action on the NMDA receptor, each consisting of five amino acids at the M3-M4 domain intersubunit interfaces.

Keywords: Addiction, Alcohol, Glutamate Receptors Ionotropic (AMPA, NMDA), Ion Channels, Receptor Structure-Function

Introduction

Unlike most drugs of abuse, ethanol produces its effects on the CNS at high (millimolar) concentrations and through actions on multiple target proteins (1, 2). One of the major targets of ethanol in the brain is the *N*-methyl-D-aspartate (NMDA) glutamate receptor (3–5). Ethanol inhibition of NMDA receptors *in vitro* is well established; ethanol inhibits NMDA receptor current (6, 7) and NMDA receptor-mediated events such as Ca^{2+} influx (8, 9), excitatory synaptic potentials (10), and neurotransmitter release (11). Studies in multiple animal species (12–17) and in humans (18–20) have demonstrated a crucial role for NMDA receptor inhibition in the subjective and behavioral effects of alcohol. The molecular sites through which

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ethanol modulates the function of the NMDA receptor, however, have been difficult to identify. The phosphorylation state of residues in the intracellular C-terminal domain can influence ethanol inhibition of the NMDA receptor (22–27), but this involves a regulation of ethanol sensitivity rather than the mechanism of ethanol action because ethanol inhibition is unchanged or enhanced by the removal of the NMDA receptor C-terminal domain (28). Using scanning mutagenesis approaches, we and others have previously identified a number of putative sites of alcohol action in the NMDA receptor M3 and M4 domains (29–33). Ronald *et al.* (32) initially demonstrated that mutations at Phe-639 in the M3 domain of the GluN1 subunit could influence ethanol inhibition of the NMDA receptor and that the characteristics of this position were consistent with a site of alcohol action. Studies from this laboratory in the GluN2A subunit found that the cognate position, Phe-637, also modulates ethanol inhibition (31) and identified two positions in the M4 domain, Met-823 and Ala-825, that regulate ethanol sensitivity (29, 30). Although the role of the latter position is still unclear, the characteristics of Met-823 fulfill some criteria for a site of alcohol action, and molecular dynamics simulations are consistent with ethanol binding to this side chain (34).

The action of ethanol on NMDA receptors involves modulation of ion channel gating rather than agonist binding (7, 35). Of the ethanol-sensitive positions identified to date, Phe-637 and Met-823 in the GluN2A subunit strongly regulate ion channel gating, as mutations at these positions alter ion channel mean open time and apparent desensitization (31, 36). In a previous study we found that these positions functionally interact with respect to ethanol sensitivity but that they do not appear to form a unitary site of alcohol action (34). This finding is consistent with a structural model of the NMDA receptor (supplemental information in Ref. 37) based upon that of the related GluA2 receptor (37). In the model, as in the GluA2 receptor structure, the M3 domains line the upper part of the channel pore. Because of the diagonal subunit arrangement in NMDA receptors (N1/N2/N1/N2) (38), the outward face of the M3 domains of one subunit type is oriented toward, and appears to form multiple interactions with, the M4 domain of the adjacent subunit of the other type. The structural model predicts the presence of four sites of alcohol action on the

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NMDA receptor, each containing five amino acids: two sites at the GluN1 M3/GluN2A M4 interfaces and two sites at the GluN1 M4/GluN2A M3 interfaces, such that GluN2A(Phe-637) would be present at one type of site and GluN2A(Met-823) at the other. In this study we report that additional residues predicted to form these sites can alter ethanol inhibition and that all side chains predicted to be in close proximity interactively regulate both ethanol inhibition and receptor kinetics. We additionally report a shift by one position of the GluN1 M4 domain relative to the proposed structural model such that a methionine is present in the initial position of the ethanol site in M4 at both sites. Based on these findings, we propose the existence of four sites of ethanol action on NMDA receptors located at the M3-M4 domain interfaces.

Experimental Procedures

Materials

All drugs and chemicals were obtained from Sigma. Chemicals used to make recording solutions were the highest purity available.

Site-directed Mutagenesis, Cell Culture, and Transfection

Site-directed mutagenesis in plasmids containing GluN1 or GluN2A subunit cDNA was performed using the QuikChange II kit (Agilent Technologies, Santa Clara, CA), and all mutants were verified by double-strand DNA sequencing. Human embryonic kidney 293 (HEK 293) cells were transfected with GluN1, GluN2A, and green fluorescent protein at a ratio of 2:2:1 using the calcium phosphate transfection kit (Invitrogen), and either 200 μm d-l-2-amino-5-phosphonovaleric acid (APV) and 100 μm ketamine or 1 mm APV was added to the culture medium to prevent excitotoxic cell death. NMDA antagonists were removed before use in experiments by extensive washing. Cells were used in experiments 18–48 h after transfection.

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Electrophysiological Recording

Whole-cell patch clamp recording was performed at room temperature using an Axopatch 1D or Axopatch 200B (Axon Instruments Inc., Foster City, CA) amplifier essentially as described previously (39). Briefly, patch-pipettes had open tip resistances of 2–8 megaohms after heat polishing; series resistances of 4–15 megaohms were compensated by 80%. Cells were voltage-clamped at −50 mV and superfused in an external recording solution containing 150 mm NaCl, 5 mm KCl, 0.2 mm $CaCl_2$, 10 mm HEPES, 10 mm glucose, and 10 mm sucrose. The ratio of added HEPES-free acid and sodium salt was calculated to result in a solution pH of 7.4 (Buffer Calculator, R. Beynon, University of Liverpool); the final pH was adjusted if necessary using HCl. Patch-pipettes were filled with a solution containing 140 mm CsCl, 2 mm Mg_4ATP, 10 mm BAPTA,[2] and 10 mm HEPES (pH 7.2). Solutions of agonists and ethanol were prepared fresh daily and applied to cells using a stepper motor-driven rapid solution exchange apparatus (Warner Instruments, Inc.) and 600-μm inner diameter square glass tubing. After obtaining a gigaohm seal, cells were lifted off the surface of the dish to increase the speed of the solution exchange; the 10–90% rise time for solution exchange under these conditions is ~1.5 ms (36). In concentration-response experiments, the order of application of the various concentrations of ethanol was randomized for each cell to eliminate time-dependent effects. Data were filtered at 2 kHz (8-pole Bessel) and acquired at 5 kHz on a computer using a DigiData interface and pClamp software (Axon Instruments).

Data Analysis

In concentration-response experiments, IC_{50} and n (slope factor) were calculated using the equation $y = E_{max}/1 + (IC_{50}/x)^n$, where y is the measured current amplitude, x is concentration, n is the slope factor, and E_{max} is the maximal current amplitude. Statistical differences among concentration-response curves were determined by comparing log transformed IC_{50} values from fits to data obtained from individual cells using analysis of variance (ANOVA).

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Significant interactions with respect to ethanol sensitivity among mutants at multiple positions were determined by two-way ANOVA of log-transformed IC_{50} values and by mutant cycle analysis. Mutant cycle analysis was performed essentially as described by Venkatachalan and Czajkowski (40). Briefly, tryptophan substitution mutations were introduced singly and in combination at positions in GluN1 and GluN2 subunits proposed to interact, and ethanol IC_{50} was determined in each mutant. The apparent interaction free energy $\Delta\Delta G_{INT}$ for mutations at two positions is the free energy difference between the parallel energies in the cycle (*i.e.* from the wild-type and either single mutant to the other single mutant and the dual mutant). Apparent interaction free energies among mutated positions were calculated using natural logarithms (ln) of ethanol IC_{50} values obtained from wild-type and mutant subunit combinations using the equation $\Delta\Delta G_{INT} = RT[\ln(WT) + \ln(mut1,mut2) - \ln(mut1) - \ln(mut2)]$. Because non-interacting positions should have an apparent interaction free energy of zero, mean values of $\Delta\Delta G_{INT}$ ± S.E. were tested for statistically significant differences from zero energy using one-sample *t* tests with degrees of freedom df = $N_{WT} + N_{MUT1} + N_{MUT2} + N_{MUT1,MUT2} - 4$, with N_X equal to the number of cells used for each combination of wild-type and mutant subunits and S.E. determined from propagated errors. Values for ethanol log IC_{50} in GluN2A tryptophan mutants at Phe-637, Ala-825, and Met-823 are those reported previously (30, 31, 39).

Time constants (τ) of deactivation were determined from fits of the current decay after the removal of glutamate (in the continued presence of glycine) to an exponential function using Clampfit (Axon Instruments). In most cells deactivation was best fitted using a bi-exponential function; in these cases, the weighted time constant is reported. For cells in which deactivation was adequately fitted by a single exponential function, this value is reported. In one mutant pair, GluN1(Leu-819) and GluN2A(Phe-637), steady-state to peak current ratios were determined as a measure of apparent desensitization. Significant interactions with respect to deactivation and steady-state to peak current ratios among mutants at multiple positions were determined by two-way ANOVA and by mutant cycle analysis, which

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was performed using the equation and analysis described above for ethanol IC_{50} values. All values are reported as means ± S.E.

Results

Single Mutations in the M3 and M4 Domains Alter Ethanol Inhibition of NMDA Receptors

Based on the reported structure of the GluA2 glutamate receptor M domains (37) and previous findings from this and another laboratory, we predicted that sites of alcohol action in the NMDA receptor would be formed by groups of 4–6 residues clustered in small regions at the M3-M4 intersubunit interfaces (Fig. 1). Our previous studies have identified three residues in these regions in the GluN2A subunit as putative sites of alcohol action, and a study from another laboratory (32) demonstrated a similar role for GluN1(Phe-639). Because tryptophan substitution has consistently produced the greatest modulation of ion channel behavior without loss of function when substituted into these domains in our previous studies (29–31, 34, 36, 39), we first tested whether tryptophan substitution at each of the remaining positions in these regions altered alcohol sensitivity. Because GluN1(Met-818), the GluN1 cognate of GluN2A(Met-823), is shifted one position relative to GluN2A(Met-823), we tested four positions in GluN1 M4. All of the tryptophan substitution mutants tested formed functional NMDA receptors (Fig. 2). All of the GluN2A tryptophan substitution mutants exhibited decreased sensitivity to ethanol, with the exception of GluN2A(Leu-824), in which the ethanol IC_{50} value was not significantly changed. In contrast, although tryptophan substitutions in GluN1 M3 decreased ethanol sensitivity, tryptophan substitution at any of the four positions in GluN1 M4 did not.

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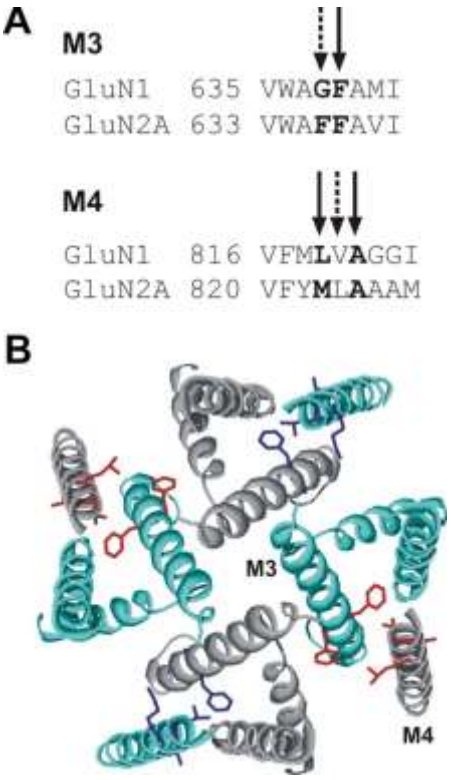

FIGURE 1. *A*, alignment of M3 and M4 domain residues in GluN1 and GluN2A subunits is shown. Alcohol-sensitive residues identified in previous studies (29–33) are denoted by *solid arrows*; adjacent residues proposed to contribute to alcohol-sensitive sites are denoted by *dashed arrows. B*, shown is a model of the NMDA receptor M domains from (37). M domains of the GluN1 subunit are shown in *gray*, and those of the GluN2A subunit are shown in *cyan*. Side chains of the five positions noted in *A* are illustrated in *red* for the GluN1 M4/GluN2A M3 interface and in *blue* for the GluN1 M3/GluN2A M4 interface.

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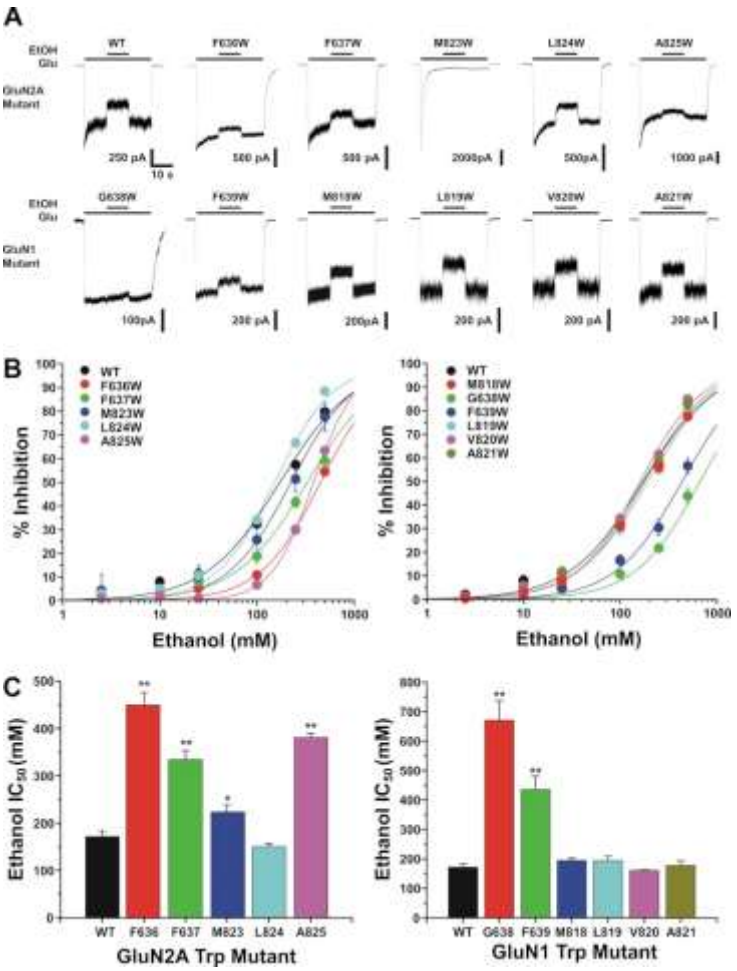

FIGURE 2. Single mutations in the M3 and M4 domains of GluN1 and GluN2A subunits can alter ethanol inhibition. *A*, records are currents activated by 10 μm glutamate in the presence of 50 μm glycine in cells expressing various mutant GluN2A (*upper*) and GluN1 (*lower*) subunits, as indicated. The current trace for F637T is from Ren *et al.* (31). *B*, shown are concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressing various single site substitution mutations in GluN2A (*left*) and GluN1 (*right*). Data are the means ± S.E. of n = 5–9 cells; *error bars* not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Experimental Procedures." *C*, graphs plot IC_{50} values for ethanol in various single site substitution mutations in GluN2A (*left*) and GluN1 (*right*). *Asterisks* indicate IC_{50} values that differed significantly from that for wild-type GluN1/GluN2A subunits (*, $p < 0.05$; **, $p < 0.01$; ANOVA followed by Dunnett's test). Values in *B* and *C* for M823T are from Ren *et al.* (30), for Leu-824Trp are from Honse *et al.* (29), for F637T are from Ren *et al.* (31), and for Ala-825Trp are from Salous *et al.* (39).

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Coexpressed GluN1 and GluN2A Mutants in the M3 and M4 Domains Alter Ethanol Inhibition of NMDA Receptors

We coexpressed GluN1 and GluN2A subunits with tryptophan substitutions at ethanol-sensitive positions in the M3-M4 domain interfaces that are predicted by the structural model to be in close proximity and determined the sensitivity of the resultant NMDA receptors to ethanol inhibition. Because of the shift of GluN1(Met-818) by one amino acid relative to its cognate in GluN2A (see above), we tested for two possible interactions with each of the GluN2A M3 alcohol-sensitive positions. We found that each of the mutant combinations tested formed functional receptors (Fig. 3). In some cases, the characteristics of the receptors, such as apparent desensitization, appeared to differ considerably from those of the mutants at either single subunit. For example, neither the GluN1(L819T) nor GluN2A(F637T) mutant subunits affected apparent desensitization when expressed with the corresponding wild-type subunit, but apparent desensitization was considerably increased when both mutants were expressed together. Ethanol IC_{50} values in the mutant combinations differed significantly from the wild-type subunits, with the exception of the GluN1(G638T)/GluN2A(M823T) combination. This latter result is of particular interest because either mutation by itself significantly increased ethanol IC_{50} (30).

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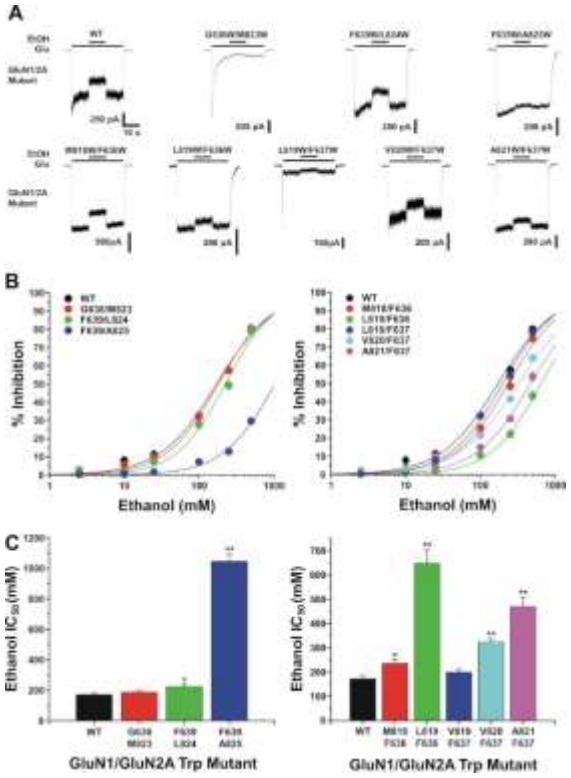

FIGURE 3. Coexpression of an NMDA receptor GluN1 or GluN2A M3 domain mutant subunit with an M4 domain mutant of the other subunit type can alter ethanol inhibition. *A*, records are currents activated by 10 μm glutamate in the presence of 50 μm glycine in cells expressing subunits with various mutations in the GluN1 M3/GluN2A M4 domains (*upper*) and the GluN1 M4/GluN2A M3 domains (*lower*) as indicated. *B*, shown are concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressing various dual-site substitution mutations in the GluN1 M3/GluN2A M4 domains (*left*) and the GluN1 M4/GluN2A M3 domains (*right*). Data are the means ± S.E. of n = 5–9 cells; *error bars* not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Experimental Procedures." *C*, graphs plot IC_{50} values for ethanol in various dual-site substitution mutations in the GluN1 M3/GluN2A M4 domains (*left*) and the GluN1 M4/GluN2A M3 domains (*right*). *Asterisks* indicate IC_{50} values that differed significantly from that for wild-type GluN1/GluN2A subunits (*, $p < 0.05$; **, $p < 0.01$; ANOVA was followed by Dunnett's test).

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Positions in the M3 and M4 Domains of GluN1 and GluN2A Subunits Interact to Regulate Ethanol Inhibition of NMDA Receptors

If the amino acid side chains at the designated positions in M3 and M4 interact with one another and if this interaction is essential in mediating the action of alcohol on the ion channel, then mutations at the two positions should influence ethanol sensitivity in a manner that is non-additive. To test this we used both two-way analysis of variance (ANOVA) on log-transformed IC_{50} values and mutant cycle analysis. Of the GluN1 M3/GluN2A M4 mutant combinations, we found an interaction between GluN1(Gly-638) and GluN2A(Met-823), as both types of analysis were statistically significant (Fig. 4 and Table 1). The results for the combination GluN1(Phe-639)/GluN2A(Leu-824) were also indicative of an interaction. Interestingly, although the mutants at positions GluN1(Phe-639) and the previously identified alcohol site GluN2A(Ala-825) markedly increased ethanol IC_{50} both individually and in combination, there was no evidence of an interaction between these two positions. For the GluN1 M4/GluN2A M3 mutant combinations, we tested for interactions of two positions with GluN2A(Phe-636) and three positions with GluN2A(Phe-637). This allowed investigating multiple possible alignments of these positions between the two domains. As was the case for the GluN1 M3/GluN2A M4 mutants, we obtained clear evidence for interactions between pairs of residues in GluN1 M4 and GluN2A M3 (Fig. 5 and Table 1). Both types of analysis indicated an interaction of GluN2A(Phe-636) in M3 with GluN1(Met-818) in M4 but not with GluN1(Leu-819) in M4. For GluN2A(Phe-637), the analysis indicated an interaction with GluN1(Leu-819) but not with GluN1(Val-820).

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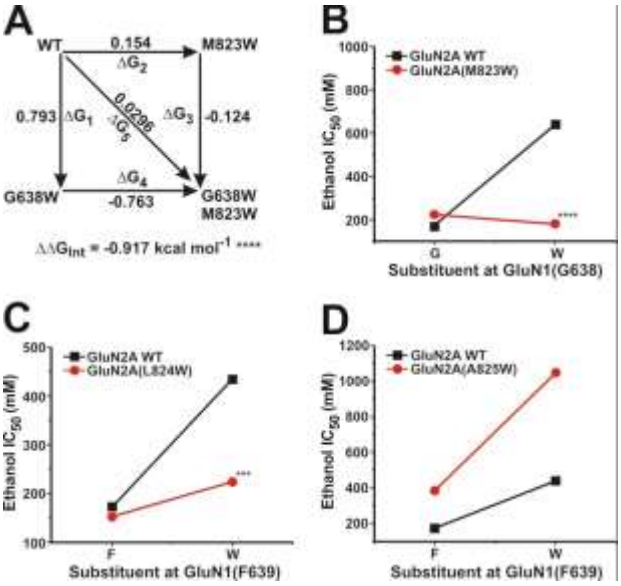

FIGURE 4. Positions in the GluN1 subunit M3 domain interact with GluN2A M4 domain residues to regulate NMDA receptor ethanol sensitivity. *A*, mutant cycle analysis of ethanol IC_{50} values for the subunit combination GluN1(Gly-638)/GluN2A(Met-823). Apparent free energy values associated with the various mutations (ΔG_X) are given in kcal mol^{-1}. *Asterisks* indicate a statistically significant difference of the apparent interaction energy $\Delta\Delta G_{INT}$ from zero energy determined using a one-sample *t* test (****, $p < 0.0001$). *B–D*, graphs plot ethanol IC_{50} values *versus* the substituent at position 638 or 639 in GluN1 for mutants at GluN2A positions 823–825 as indicated. *Asterisks* indicate significant interactions detected using log-transformed IC_{50} values (***, $p < 0.005$; ****, $p < 0.0001$; two-way ANOVA). IC_{50} values for M823T are from Ren *et al.* (30), for Leu-824Trp are from Honse *et al.* (29), and for Ala-825Trp are from Salous *et al.* (39).

TABLE 1

Mutant cycle analysis of ethanol concentration response

Values of ΔG_X in kcal mol^{-1} are RT[ln(NMDAR1 IC_{50}) − ln(NMDAR2 IC_{50})], where NMDAR1 and NMDAR2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (N1, GluN1 mutant/GluN2A wild type; N2A, GluN1 wild type/GluN2A mutant; N1/N2A, GluN1 mutant/GluN2A mutant). Values of apparent free energy $\Delta\Delta G_{INT}$ in kcal mol^{-1} are the means ± S.E. $\Delta\Delta G_{INT}$, degrees of freedom (df), and statistical significance of $\Delta\Delta G_{INT}$ were determined as described under "Experimental Procedures."

Mutant pair	ΔG_1	ΔG_2	ΔG_3	ΔG_4	ΔG_5	$\Delta\Delta G_{INT}$	df	Significance
(GluN1/GluN2A)	WT → N1	WT → N2A	N2A → N1/N2A	N1 → N1/N2A	WT → N1/N2A			
G638W/M823W	0.793	0.154	−0.124	−0.763	0.0296	−0.917 ± 0.149	23	$p < 0.0001$
F639W/L824W	0.539	−0.0769	0.231	−0.385	0.154	−0.308 ± 0.144	21	$p < 0.05$
F639W/A825W	0.539	0.473	0.598	0.533	1.07	0.0592 ± 0.132	19	$p > 0.05$
M818W/F636W	0.0769	0.571	−0.381	0.112	0.189	−0.458 ± 0.114	17	$p < 0.001$
L819W/F636W	0.0710	0.571	0.200	0.704	0.775	0.134 ± 0.143	20	$p > 0.05$
L819W/F637W	0.0710	0.389	−0.307	0.0118	0.0829	−0.378 ± 0.136	22	$p < 0.05$
V820W/F637W	−0.0355	0.389	−0.0166	0.408	0.373	0.0189 ± 0.102	22	$p > 0.05$
A821W/F637W	0.0178	0.389	0.202	0.574	0.592	0.185 ± 0.144	22	$p > 0.05$

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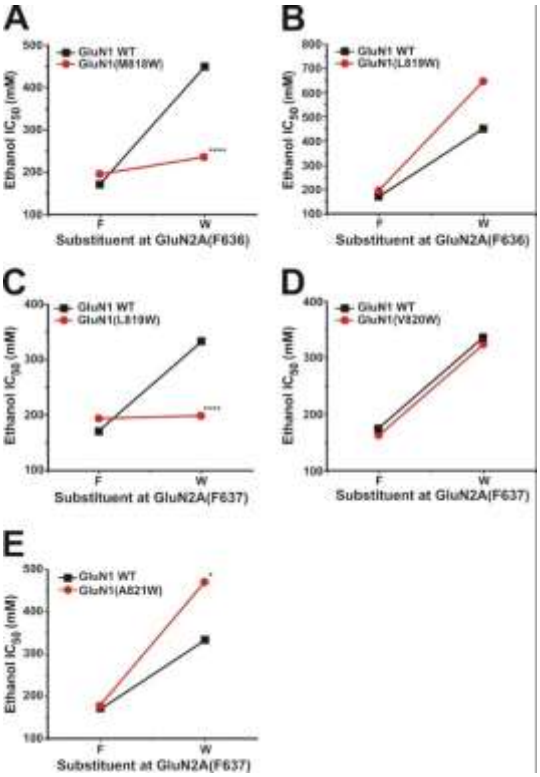

FIGURE 5. Positions in the GluN2A subunit M3 domain interact with GluN1 M4 domain residues to regulate NMDA receptor ethanol sensitivity. Graphs plot ethanol IC_{50} values *versus* the substituent at position 636 (*A* and *B*) or 637 (*C*–*E*) in GluN2A for mutants at GluN1 positions 818–821, as indicated. *Asterisks* indicate significant interactions detected using log-transformed IC_{50} values (*, $p < 0.05$; ****, $p < 0.0001$; two-way ANOVA). The IC_{50} value for F637T is from Ren *et al.* (31).

Positions in the M3 and M4 Domains of GluN1 and GluN2A Subunits Interact to Regulate NMDA Receptor Kinetics

We and others have previously shown that mutations at alcohol-sensitive positions in M3 and M4 of the GluN1 and GluN2A subunits can alter NMDA receptor kinetics (31–34, 36). If amino acid side chains at two positions in the M3 and M4 domains interact and if one or both of these positions are important in regulating ion channel function, then mutations at the two positions should affect relevant measures of receptor-ion channel function in a non-additive manner. We accordingly tested for interactions among the positions in M3 and M4 of the GluN1 and GluN2A subunits with respect to NMDA receptor

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deactivation, or decay of current after rapid removal of glutamate using two-way analysis of variance and mutant cycle analysis. Time constants of deactivation differed among the various mutants, and we observed significant interactions with respect to deactivation among pairs of positions in M3 and M4 (Fig. 6 and Table 2). For the GluN1 M4/GluN2A M3 mutant subunit combinations, we found that GluN2A(Phe-636) interacted with GluN1(Met-818) but not with GluN1(Leu-819) and that GluN2A(Phe-637) interacted with GluN1(Leu-819) but not with GluN1(Val-820). In contrast, both GluN1 M3 positions studied appeared to interact at least weakly with two positions in GluN2A M4. GluN1(Gly-638) interacted with both Met-823 and Leu-824 in GluN2A, and GluN1(Phe-639) interacted strongly with GluN2A(Leu-824). GluN1(Phe-639) also appeared to interact weakly with GluN2A(Ala-825), as a significant interaction was detected by two-way ANOVA but not by mutant cycle analysis.

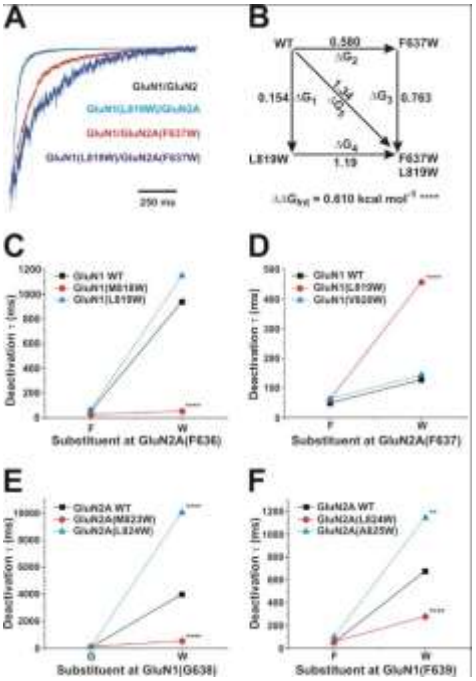

FIGURE 6. Positions in the GluN1 and GluN2A subunit M3 and M4 domains interact to regulate NMDA receptor deactivation rate. *A*, records show decay of currents activated by 10 μm glutamate and 50 μm glycine after rapid removal of glutamate in cells expressing subunits with various mutations. Current amplitudes are normalized to that of the wild-type subunits. *Dashed lines* are double exponential fits to the data. *B*, shown is mutant cycle analysis of deactivation time constants for the subunit combination GluN2A(Phe-637)/GluN1(Leu-819). Apparent free energy values

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associated with the various mutations ((ΔG_X) are given in kcal mol^{-1}. *Asterisks* indicate a statistically significant difference of the apparent interaction energy $\Delta\Delta G_{INT}$ from zero energy determined using a one-sample *t* test (****, $p < 0.0001$). *C–F*, graphs plot weighted deactivation time constants *versus* the substituent at position 636 (*C*) or 637 (*D*) in GluN2A for mutants at GluN1 positions 818–821 and the substituent at position 638 (*E*) or 639 (*F*) in GluN1 for mutants at GluN2A positions 823–825, as indicated. *Asterisks* indicate significant interactions (*, $p < 0.05$; ****, $p < 0.0001$; two-way ANOVA).

TABLE 2

Mutant cycle analysis of deactivation

Values of ΔG_X in kcal mol^{-1} are RT[lnNMDAR1 IC_{50}) − lnNMDAR2 IC_{50})], where NMDAR1 and NMDAR2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild type; N1, GluN1 mutant/GluN2A wild type; N2A, GluN1 wild type/GluN2A mutant; N1/N2A, GluN1 mutant/GluN2A mutant). Values of apparent free energy $\Delta\Delta G_{INT}$ in kcal mol^{-1} are the means ± S.E. $\Delta\Delta G_{INT}$, degrees of freedom (df), and statistical significance of $\Delta\Delta G_{INT}$ were determined as described under "Experimental Procedures."

Mutant pair	ΔG_1	ΔG_2	ΔG_3	ΔG_4	ΔG_5	$\Delta\Delta G_{INT}$	df	Significance
(GluN1/GluN2A)	WT → N1	WT → N2A	N2A → N1/N2A	N1 → N1/N2A	WT → N1/N2A			
G638W/M823W	2.62	0.420	1.02	−1.18	1.44	−1.60 ± 0.145	23	$p < 0.0001$
G638W/L824W	2.62	0.178	2.99	0.550	3.17	0.373 ± 0.108	21	$p < 0.005$
F639W/L824W	1.55	0.178	0.834	−0.539	1.01	−0.716 ± 0.150	27	$p < 0.0001$
F639W/A825W	1.55	0.397	1.47	0.320	1.87	−0.0769 ± 0.109	30	$p > 0.05$
M818W/F636W	−0.393	1.75	−1.81	0.340	−0.0532	−1.412 ± 0.142	22	$p < 0.0001$
L819W/F636W	0.154	1.75	0.118	1.72	1.87	−0.0355 ± 0.118	24	$p > 0.05$
L819W/F637W	0.154	0.580	0.763	1.19	1.34	0.610 ± 0.084	20	$p < 0.0001$
V820W/F637W	0.107	0.580	0.0786	0.552	0.659	−0.0279 ± 0.168	18	$p > 0.05$

In addition to interactions affecting deactivation, we also observed a significant interaction between GluN1(Leu-819) and GluN2A(Phe-637) with respect to apparent desensitization of glutamate-activated current (Fig. 7), as assessed by using steady-state to peak current ratios. For GluN1(Ala-821), two-way analysis of variance indicated a deviation from parallelism consistent with a weak interaction with GluN2A(Phe-637) (two-way ANOVA, $p < 0.05$); however, results of mutant cycle analysis were not consistent with an interaction between these positions.

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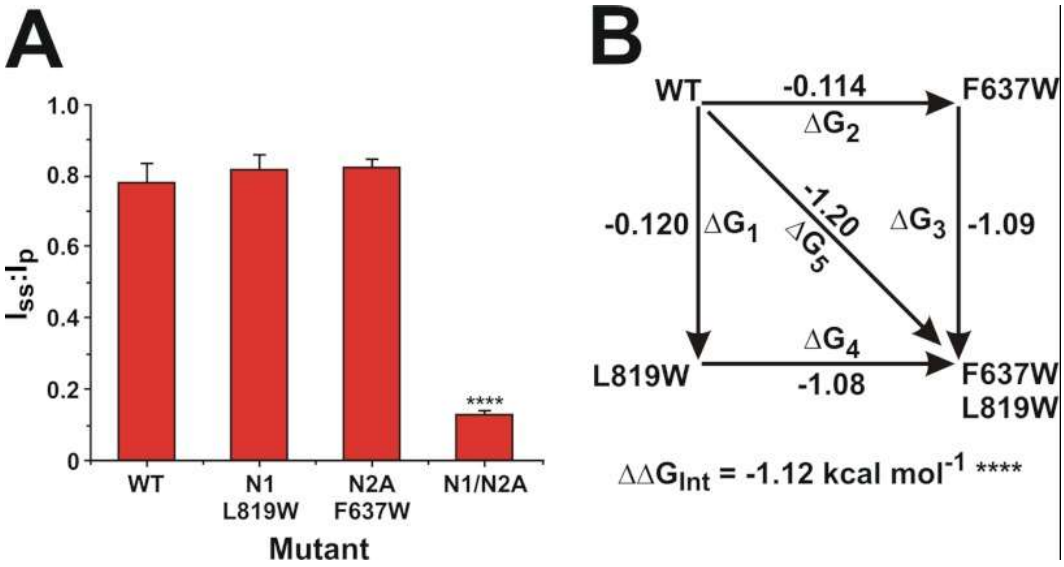

FIGURE 7.

Positions GluN1(Leu-819) and GluN2A(Phe-637) interact to regulate NMDA receptor apparent desensitization. *A*, the *bar graph* shows values of steady-state to peak current ratio (I_{ss}:I_p) for currents activated by 10 μm glutamate and 50 μm glycine in cells expressing wild-type (*WT*), GluN1(Leu-819), and GluN2A(Phe-637) subunits. I_{ss}:I_p in the dual mutant (N1/N2A) differed significantly from that of all other subunit combinations (one-way ANOVA and Tukey's test; ****, $p < 0.0001$). *B*, shown is mutant cycle analysis of steady-state to peak current ratios for the subunit combination GluN2A(Phe-637)/GluN1(Leu-819). Apparent free energy values associated with the various mutations (ΔG_X) are given in kcal mol^{-1}. *Asterisks* indicate a statistically significant difference of the apparent interaction energy $\Delta\Delta G_{INT}$ from zero energy determined using a one-sample *t* test (****, $p < 0.0001$).

Discussion

We and others have previously identified a small number of positions in the GluN1 M3 domain and GluN2A M3 and M4 domains that influence NMDA receptor alcohol sensitivity: Phe-639 in M3 of GluN1 (32), the cognate position, Phe-637, in M3 of the GluN2A subunit (31), and two positions, Met-823 and Ala-825, in the GluN2A M4 domain (29, 30, 36). A structural model of the NMDA receptor M domains, based on the solved x-ray crystallographic structure of the highly homologous GluA2 subunit (37), places these residues in the M3 and M4 domains of adjacent subunits in close proximity, with the M3 residues of one subunit closely apposed to the M4 residues of the other subunit type. The proximity of two of these residues, the

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GluN1(Phe-639) and GluN2A(Ala-825), has been recently reported by the Woodward laboratory (41). The NMDA receptor structural model of Sobolevsky *et al.* (37) also predicts the involvement of nearby residues. In earlier studies, Smothers and Woodward (33) reported an interaction between GluN1(Phe-639) and GluN1 M4 domain residues with respect to ethanol inhibition, and our laboratory reported that GluN2A Phe-637 and Met-823 could interactively regulate ethanol sensitivity but that these two positions appeared to form separate sites of alcohol action (34). In this study we demonstrate that pairs of residues in the M3 domain of one subunit type and the M4 of the other subunit type interact to regulate ethanol sensitivity and ion channel function and appear to contribute to a unitary site of ethanol action (Fig. 8).

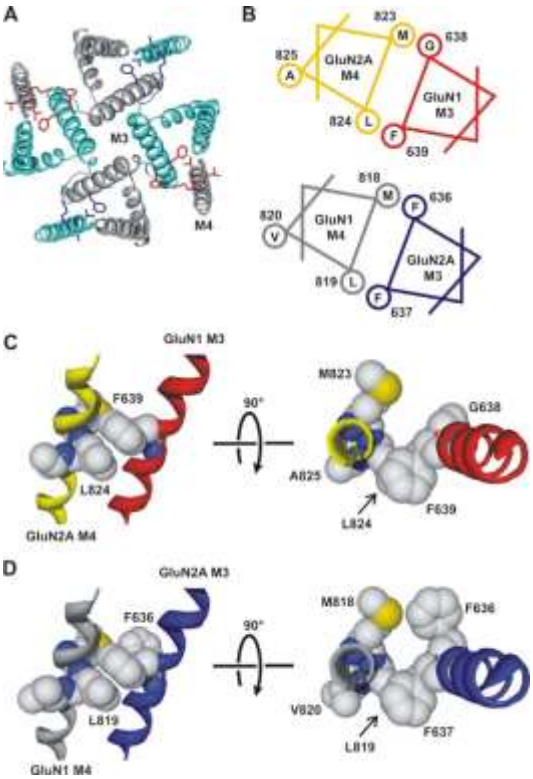

FIGURE 8. The NMDA receptor M domains. *A*, model of the NMDA receptor M domains from (37) incorporating the shift by one position of GluN1 M4. M domains of the GluN1 subunit are shown in *gray*, and those of the GluN2A subunit are shown in *cyan*. Side chains in the five positions shown to modulate ethanol sensitivity are illustrated in *red* for the GluN1 M4/GluN2A M3 interface and in *blue* for the GluN1 M3/GluN2A M4 interface. *B*, shown are helical wheel plots of the regions of the GluN1

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and GluN2A M3 and M4 domains containing ethanol-sensitive positions. *C*, shown is a molecular model of the ethanol site formed by the GluN2A subunit M4 domain (*yellow*) and GluN1 subunit M3 domain (*red*). *D*, shown is a molecular model of the ethanol site formed by the GluN1 subunit M4 domain (*gray*) and GluN2A subunit M3 domain (*blue*).

Although this study does not directly address the manner in which ethanol binds to its target sites on the protein, our results demonstrate that mutations at specific positions in M3 and M4 strongly influence ethanol sensitivity in a non-additive manner. These observations are consistent with the interpretation that the side chains at these positions interact with one another and that these interactions are important in mediating the action of alcohol. In this study we used both two-way analysis of variance and mutant cycle analysis of log-transformed ethanol IC_{50} values to test for interactions among mutated positions. Although half-maximal concentration values are complex and represent multiple kinetic rates, previous studies have shown the suitability of mutant cycle analysis of complex macroscopic measures to indicate side-chain interactions (40, 42, 43). In addition, ethanol IC_{50} values may represent fewer kinetic rates compared with agonist EC_{50} values, because the main action of ethanol on NMDA receptor kinetics is to decrease mean open time (7), whereas agonist EC_{50} values depend on multiple microscopic rates underlying both agonist binding and ion channel gating (44).

Ion channel gating in glutamate receptors is regulated by both the M3 (45–51) and M4 (36, 52, 53) domains. Mutations at a number of alcohol-sensitive positions in M3 and M4 of the GluN1 and GluN2A subunits can alter multiple NMDA receptor kinetic parameters, such as desensitization, mean open time, and agonist affinity (31–34, 36), indicating that these positions participate in regulation of receptor-ion channel function. In this study we observed that the rate of deactivation of current after rapid removal of agonist was altered in a number of single and dual mutants. We previously reported that GluN2A(Met-823) regulates receptor desensitization and that mutations at this position can affect apparent affinity of the receptor for glutamate by trapping the agonist to the closed, desensitized state of the receptor-ion channel (36). This mechanism cannot account for the changes in deactivation caused by mutations at the majority of the

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positions tested in this study, because desensitization was unaffected in most of the mutants tested. Nevertheless, because these mutations are in domains regulating gating and are far removed from the agonist binding domains, the alterations we observed in apparent agonist affinity most likely result from alterations in gating caused by the mutations. The positions studied in M3 and M4 thus appear to interactively regulate gating, because we observed strong intersubunit interactions among positions in the M3 and M4 domains with respect to deactivation rate that generally agreed well with the results obtained in ethanol concentration-response experiments. However, the interactions in deactivation rate between the two positions in GluN1 M3 and the three positions in GluN2A M4 were somewhat ambiguous, as each position in GluN1 M3 significantly interacted with two positions in GluN2A M4 as determined by two-way analysis of variance. Results of mutant cycle analysis, however, showed that GluN1(G638) interacts with GluN2A(Met-823) but not GluN2A(Leu-824). Although mutant cycle analysis showed significant interactions of GluN1(Phe-639) with both GluN2A(Leu-824) and GluN2A(Ala-825), the apparent free energy of interaction was >4-fold greater for the former *versus* the latter pair. The weaker interactions we observed between GluN1 M3 and GluN2A M4 positions may thus reflect functional interactions, resulting from long-distance alterations in conformation, as we have observed previously for GluN2A M3 and M4 positions (34), but may also result from shorter-lived conformational changes associated with ion channel gating.

For one pair of positions, GluN1(Leu-819) and GluN2A(Phe-637), we also observed an interaction with respect to apparent desensitization. NMDA receptors exhibit multiple forms of apparent desensitization or macroscopic current decay in the prolonged presence of agonist (54); the conditions we used in this study were designed to minimize the influence of forms of current decay other than true desensitization, which refers to sojourns of the liganded receptor in one or more long-lived closed states (55–57) and thus is a form of ion channel gating. In the NMDA receptor, the M3 domain is primarily responsible for gating of the ion channel (46–48, 51), and we and others have shown that the M4 domain regulates gating of the ion channel (36, 52). Furthermore, alcohol-sensitive positions in both the

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M3 and M4 domains of the GluN2A subunit can influence both channel open times, reflecting changes in ion channel closing rates, and macroscopic desensitization, which could result from changes in multiple microscopic kinetic rates (31, 34, 36). For two of the mutant subunits in this study, GluN1(L819T) and GluN2A(F637T), apparent desensitization of glutamate-activated current was unaltered when either was expressed with the corresponding wild-type subunit but was markedly increased when these mutant subunits were coexpressed. We observed a significant interaction between these two positions using mutant cycle analysis of steady-state to peak current ratio, a measure of macroscopic desensitization. Our results demonstrate both that the side chains at these positions are able to interact and that the GluN1 M4/GluN2A M3 interface, at least when both positions bear a tryptophan substitution, is able to regulate ion channel gating. Because ion channel opening appears to result from the movement of the M3 and M4 domains relative to each other (37, 58), these results may indicate that the altered interaction at these positions in the dual mutant stabilizes a desensitized state by opposing the conformational change of M3 and M4 associated with channel opening.

Our results generally agree with the predictions of the model of Sobolevsky *et al.* (37) based on the GluA2 subunit. In the case of the GluN1 M4 domain, however, our results point to a shift by one position in the proposed structural model. Compared with the GluN2A subunit M4 domain, the GluN1 subunit lacks a homologous tyrosine before a methionine at position 818. Sobolevsky *et al.* (37) reasonably proposed, based on the homology of the remainder of M4, that the preceding phenylalanine residues in GluN1 and GluN2A would be aligned such that the leucine at position 819 would interact with Phe-636 in the GluN2A subunit. We found, however, that GluN2A(Phe-636) significantly interacts with GluN1(Met-818) but not with GluN1(Leu-819) and that GluN2A(Phe-637) significantly interacts with GluN1(Leu-819). In addition, our observation of a significant interaction between GluN1(L819T) and GluN2A(F637T) with respect to apparent desensitization is consistent with this interpretation and provides further support for this alignment using a measure that is not dependent upon the presence of ethanol.

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The results of this study also show that the amino acid immediately preceding the previously identified position in the M3 domain in both the GluN1 and GluN2A subunits powerfully influences alcohol sensitivity and that the cognate positions in M4 of the GluN1 subunit do not by themselves appear to regulate ethanol sensitivity. The former result was not observed previously in the original study from the Woodward laboratory in which the adjacent GluN1(Phe-639) was identified as an alcohol-sensitive position (32). Furthermore, the effect of tryptophan substitution at GluN1(Phe-639) in this study was opposite to that reported in Ronald *et al.* (32). In the present study ethanol sensitivity was significantly decreased by tryptophan substitution at GluN1(Phe-639), whereas in the previous study, it was unchanged or slightly increased by tryptophan substitution but was significantly decreased by alanine substitution. The reason for this discrepancy is not immediately clear. This study was performed in HEK 293 cells, whereas the previous study was performed in *Xenopus* oocytes. However, a more recent study from the same laboratory using HEK 293 cells found that alanine substitution at GluN1(Phe-639) markedly decreased ethanol inhibition (33). The same study did not report the effects of tryptophan substitution at this position but observed that substitution of other amino acids with large side chains, such as tyrosine, had no effect on ethanol inhibition. Further studies will thus be required to resolve these discrepant findings. In contrast, our findings regarding single tryptophan substitutions at positions in M4 of GluN1 agree with the observations of Woodward and Smothers (33). In both studies, despite the importance of the cognate positions in GluN2A in alcohol action, single substitutions at these positions in the GluN1 M4 domain had no effect on ethanol sensitivity. These results are surprising given the interaction of these positions with the M3 domain of GluN2A. Further studies involving additional substitutions at these positions in GluN1 M4 will be required to clarify their role in alcohol inhibition of NMDA receptors.

Results of studies on ethanol-sensitive positions in the NMDA receptor M3 and M4 domains (29–33) taken together with the ionotropic glutamate receptor structural model of Sobolevsky *et al.* (37) predict two types of sites of ethanol action: GluN1 M3/GluN2 M4, and GluN1 M4/GluN2 M3. Each of these sites consists of two amino

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acid positions in the M3 domain and three amino acid positions in the M4 domain. The results of this study indicate that these sites are not equivalent; mutation of the M3 domain residues in either subunit significantly alters ethanol IC_{50} values, whereas mutation of the M4 domain residues in the GluN2A, but not GluN1, subunit alters ethanol IC_{50} values. This latter result may arise from the marked differences in kinetic behavior attributed to the two types of subunits (21) and further suggests that although the GluN1 and GluN2 subunits contribute to both types of ethanol-sensitive sites, the roles of the M3 and M4 domains in forming these sites is not identical in both subunit types. The greater effect of mutations at sites in the M3 domain compared with those in the M4 domain most probably also reflects the importance of the M3 domain in ion channel gating (47, 48), whereas the influence of the M4 domain on gating is indirect and mediated by its interaction with the M3 domain (36). One surprising insight provided by the structural model is that GluN2A(Ala-825), which powerfully regulates alcohol sensitivity (29, 34), is not predicted to interact with another M domain side chain but rather is oriented toward the surrounding membrane lipids. Unless this residue interacts with another membrane protein, as has been shown for a residue approximately one c-helical turn above this position in an AMPA receptor (53), this may suggest an interaction with the membrane lipid such that both protein-protein and protein-lipid interactions could regulate ethanol action. Interestingly, tryptophan substitution at the cognate position in GluN1, Val-820, had no effect on ethanol IC_{50}, perhaps because of the greater hydrophobicity of the valine at this position compared with alanine at GluN2A(Ala-825). Also of interest is that tryptophan substitution at GluN2A(Leu-824), which we found to interact strongly with GluN1(Phe-639) and which is predicted by the model to be closely apposed to this side chain, did not by itself have any influence on ethanol sensitivity.

In summary, taking the results of this study together with those of previous studies, we propose the existence of four sites of ethanol action on NMDA receptors located at the M3-M4 domain interfaces. We previously demonstrated that mutations at individual positions in these sites can regulate ion channel gating; it will be of interest in future

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studies to determine how the amino acid side chains constituting these sites interact to influence ion channel gating and function.

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[2]The abbreviations used are:

BAPTA 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,N′-tetraacetic acid
ANOVA analysis of variance.

References

1. Franks N. P., Lieb W. R. (1994) Molecular and cellular mechanisms of anesthesia. Nature 367, 607–614
2. Peoples R. W., Li C., Weight F. F. (1996) Lipid versus protein theories of alcohol action in the nervous system. Annu. Rev. Pharmacol. Toxicol. 36, 185–201
3. Krystal J. H., Petrakis I. L., Mason G., Trevisan L., D'Souza D. C. (2003) *N*-Methyl-D-aspartate glutamate receptors and alcoholism. Reward, dependence, treatment, and vulnerability. Pharmacol. Ther. 99, 79–94
4. Peoples R. W. (2003) in Glutamate and Addiction (Herman B. H., Frankenheim J., Litten R. Z., Sheridan P. H., Weight F. F., Zukin S. R., editors. , eds) pp. 343–356, Humana Press Inc., Totowa, NJ
5. Woodward J. J. (1999) Ionotropic glutamate receptors as sites of action for ethanol in the brain. Neurochem. Int. 35, 107–113
6. Lovinger D. M., White G., Weight F. F. (1989) Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science 243, 1721–1724
7. Wright J. M., Peoples R. W., Weight F. F. (1996) Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons. Brain Res. 738, 249–256
8. Hoffman P. L., Rabe C. S., Moses F., Tabakoff B. (1989) *N*-Methyl-D-aspartate receptors and ethanol. inhibition of calcium flux and cyclic GMP production. J. Neurochem. 52, 1937–1940
9. Dildy J. E., Leslie S. W. (1989) Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells. Brain Res. 499, 383–387
10. Lovinger D. M., White G., Weight F. F. (1990) NMDA receptor-mediated synaptic excitation selectively inhibited by ethanol in hippocampal slice from adult rat. J. Neurosci. 10, 1372–1379

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11. Göthert M., Fink K. (1989) Inhibition of *N*-methyl-D-aspartate (NMDA)- and l-glutamate-induced noradrenaline and acetylcholine release in the rat brain by ethanol. Naunyn-Schmiedebergs Arch. Pharmacol. 340, 516–521
12. Grant K. A., Knisely J. S., Tabakoff B., Barrett J. E., Balster R. L. (1991) Ethanol-like discriminative stimulus effects of non-competitive *N*-methyl-D-aspartate antagonists. Behav. Pharmacol. 2, 87–95
13. Balster R. L., Grech D. M., Bobelis D. J. (1992) Drug discrimination analysis of ethanol as an *N*-methyl-D-aspartate receptor antagonist. Eur. J. Pharmacol. 222, 39–42
14. Grant K. A., Colombo G. (1993) Discriminative stimulus effects of ethanol. Effect of training dose on the substitution of *N*-methyl-D-aspartate antagonists. J. Pharmacol. Exp. Ther. 264, 1241–1247
15. Sanger D. J. (1993) Substitution by NMDA antagonists and other drugs in rats trained to discriminate ethanol. Behav. Pharmacol. 4, 523–528
16. Schechter M. D., Meehan S. M., Gordon T. L., McBurney D. M. (1993) The NMDA receptor antagonist MK-801 produces ethanol-like discrimination in the rat. Alcohol 10, 197–201
17. Hodge C. W., Cox A. A. (1998) The discriminative stimulus effects of ethanol are mediated by NMDA and $GABA_A$ receptors in specific limbic brain regions. Psychopharmacology 139, 95–107
18. Dickerson D., Pittman B., Ralevski E., Perrino A., Limoncelli D., Edgecombe J., Acampora G., Krystal J. H., Petrakis I. (2010) Ethanol-like effects of thiopental and ketamine in healthy humans. J. Psychopharmacol. 24, 203–211
19. Krystal J. H., Petrakis I. L., Webb E., Cooney N. L., Karper L. P., Namanworth S., Stetson P., Trevisan L. A., Charney D. S. (1998) Dose-related ethanol-like effects of the NMDA antagonist, ketamine, in recently detoxified alcoholics. Arch. Gen. Psychiatry 55, 354–360
20. Krupitsky E. M., Neznanova O., Masalov D., Burakov A. M., Didenko T., Romanova T., Tsoy M., Bespalov A., Slavina T. Y., Grinenko A. A., Petrakis I. L., Pittman B., Gueorguieva R., Zvartau E. E., Krystal J. H. (2007) Effect of memantine on cue-induced alcohol craving in recovering alcohol-dependent patients. Am. J. Psychiatry 164, 519–523
21. Banke T. G., Traynelis S. F. (2003) Activation of NR1/NR2B NMDA receptors. Nat. Neurosci. 6, 144–152
22. Alvestad R. M., Grosshans D. R., Coultrap S. J., Nakazawa T., Yamamoto T., Browning M. D. (2003) Tyrosine dephosphorylation and ethanol inhibition of *N*-methyl-D-aspartate receptor function. J. Biol. Chem. 278, 11020–11025

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***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

23. Hicklin T. R., Wu P. H., Radcliffe R. A., Freund R. K., Goebel-Goody S. M., Correa P. R., Proctor W. R., Lombroso P. J., Browning M. D. (2011) Alcohol inhibition of the NMDA receptor function, long term potentiation, and fear learning requires striatal-enriched protein tyrosine phosphatase. Proc. Natl. Acad. Sci. U.S.A. 108, 6650–6655
24. Miyakawa T., Yagi T., Kitazawa H., Yasuda M., Kawai N., Tsuboi K., Niki H. (1997) Fyn-kinase as a determinant of ethanol sensitivity. Relation to NMDA-receptor function. Science 278, 698–701
25. Anders D. L., Blevins T., Sutton G., Swope S., Chandler L. J., Woodward J. J. (1999) Fyn tyrosine kinase reduces the ethanol inhibition of recombinant NR1/NR2A but not NR1/NR2B NMDA receptors expressed in HEK 293 cells. J. Neurochem. 72, 1389–1393
26. Maldve R. E., Zhang T. A., Ferrani-Kile K., Schreiber S. S., Lippmann M. J., Snyder G. L., Fienberg A. A., Leslie S. W., Gonzales R. A., Morrisett R. A. (2002) DARPP-32 and regulation of the ethanol sensitivity of NMDA receptors in the nucleus accumbens. Nat. Neurosci. 5, 641–648
27. Yaka R., Tang K. C., Camarini R., Janak P. H., Ron D. (2003) Fyn kinase and NR2B-containing NMDA receptors regulate acute ethanol sensitivity but not ethanol intake or conditioned reward. Alcohol Clin. Exp. Res. 27, 1736–1742
28. Peoples R. W., Stewart R. R. (2000) Alcohols inhibit *N*-methyl-D-aspartate receptors via a site exposed to the extracellular environment. Neuropharmacology 39, 1681–1691
29. Honse Y., Ren H., Lipsky R. H., Peoples R. W. (2004) Sites in the fourth membrane-associated domain regulate alcohol sensitivity of the NMDA receptor. Neuropharmacology 46, 647–654
30. Ren H., Honse Y., Peoples R. W. (2003) A site of alcohol action in the fourth membrane-associated domain of the NMDA receptor. J. Biol. Chem. 278, 48815–48820
31. Ren H., Salous A. K., Paul J. M., Lipsky R. H., Peoples R. W. (2007) Mutations at F637 in the NMDA receptor NR2A subunit M3 domain influence agonist potency, ion channel gating, and alcohol action. Br. J. Pharmacol. 151, 749–757
32. Ronald K. M., Mirshahi T., Woodward J. J. (2001) Ethanol inhibition of *N*-methyl-D-aspartate receptors is reduced by site-directed mutagenesis of a transmembrane domain phenylalanine residue. J. Biol. Chem. 276, 44729–44735
33. Smothers C. T., Woodward J. J. (2006) Effects of amino acid substitutions in transmembrane domains of the NR1 subunit on the ethanol inhibition of recombinant *N*-methyl-D-aspartate receptors. Alcohol Clin. Exp. Res. 30, 523–530

Journal of Biological Chemistry, Vol. 287, No. 33 (August 2012): pg. 27302-27312. DOI. This article is © American Society for Biochemistry and Molecular Biology and permission has been granted for this version to appear in e-Publications@Marquette. American Society for Biochemistry and Molecular Biology does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Society for Biochemistry and Molecular Biology.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

34. Ren H., Salous A. K., Paul J. M., Lamb K. A., Dwyer D. S., Peoples R. W. (2008) Functional interactions of alcohol-sensitive sites in the *N*-methyl-D-aspartate receptor M3 and M4 domains. J. Biol. Chem. 283, 8250–8257
35. Peoples R. W., White G., Lovinger D. M., Weight F. F. (1997) Ethanol inhibition of *N*-methyl-D-aspartate-activated current in mouse hippocampal neurones. Whole-cell patch clamp analysis. Br. J. Pharmacol. 122, 1035–1042
36. Ren H., Honse Y., Karp B. J., Lipsky R. H., Peoples R. W. (2003) A site in the fourth membrane-associated domain of the *N*-methyl-D-aspartate receptor regulates desensitization and ion channel gating. J. Biol. Chem. 278, 276–283
37. Sobolevsky A. I., Rosconi M. P., Gouaux E. (2009) X-ray structure, symmetry. and mechanism of an AMPA subtype glutamate receptor. Nature 462, 745–756
38. Salussolia C. L., Prodromou M. L., Borker P., Wollmuth L. P. (2011) Arrangement of subunits in functional NMDA receptors. J. Neurosci. 31, 11295–11304
39. Salous A. K., Ren H., Lamb K. A., Hu X. Q., Lipsky R. H., Peoples R. W. (2009) Differential actions of ethanol and trichloroethanol at sites in the M3 and M4 domains of the NMDA receptor GluN2A (NR2A) subunit. Br. J. Pharmacol. 158, 1395–1404
40. Venkatachalan S. P., Czajkowski C. (2008) A conserved salt bridge critical for $GABA_A$ receptor function and loop C dynamics. Proc. Natl. Acad. Sci. U.S.A. 105, 13604–13609
41. Xu M., Smothers C. T., Trudell J., Woodward J. J. (2012) Ethanol inhibition of spontaneously active *N*-methyl-D-aspartate receptors. J. Pharmacol. Exp. Ther. 340, 218–226
42. Kash T. L., Jenkins A., Kelley J. C., Trudell J. R., Harrison N. L. (2003) Coupling of agonist binding to channel gating in the $GABA_A$ receptor. Nature 421, 272–275
43. Laha K. T., Wagner D. A. (2011) A state-dependent salt bridge interaction exists across the β/α intersubunit interface of the GABAA receptor. Mol. Pharmacol. 79, 662–671
44. Colquhoun D. (1998) Binding, gating, affinity and efficacy. The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. Br. J. Pharmacol. 125, 924–947
45. Blanke M. L., VanDongen A. M. (2008) The NR1 M3 domain mediates allosteric coupling in the *N*-methyl-D-aspartate receptor. Mol. Pharmacol. 74, 454–465
46. Chang H. R., Kuo C. C. (2008) The activation gate and gating mechanism of the NMDA receptor. J. Neurosci. 28, 1546–1556

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47. Jones K. S., VanDongen H. M., VanDongen A. M. (2002) The NMDA receptor M3 segment is a conserved transduction element coupling ligand binding to channel opening. J. Neurosci. 22, 2044–2053
48. Kohda K., Wang Y., Yuzaki M. (2000) Mutation of a glutamate receptor motif reveals its role in gating and d2 receptor channel properties. Nat. Neurosci. 3, 315–322
49. Qian A., Johnson J. W. (2002) Channel gating of NMDA receptors. Physiol. Behav. 77, 577–582
50. Sobolevsky A. I., Beck C., Wollmuth L. P. (2002) Molecular rearrangements of the extracellular vestibule in NMDAR channels during gating. Neuron 33, 75–85
51. Yuan H., Erreger K., Dravid S. M., Traynelis S. F. (2005) Conserved structural and functional control of *N*-methyl-D-aspartate receptor gating by transmembrane domain M3. J. Biol. Chem. 280, 29708–29716
52. Schorge S., Colquhoun D. (2003) Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. J. Neurosci. 23, 1151–1158
53. Terhag J., Gottschling K., Hollmann M. (2010) The transmembrane domain C of AMPA receptors is critically involved in receptor function and modulation. Front. Mol. Neurosci. 3, 117.
54. Dingledine R., Borges K., Bowie D., Traynelis S. F. (1999) The glutamate receptor ion channels. Pharmacol. Rev. 51, 7–61
55. Erreger K., Dravid S. M., Banke T. G., Wyllie D. J., Traynelis S. F. (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. J. Physiol. 563, 345–358
56. Schorge S., Elenes S., Colquhoun D. (2005) Maximum likelihood fitting of single channel NMDA activity with a mechanism composed of independent dimers of subunits. J. Physiol. 569, 395–418
57. Auerbach A., Zhou Y. (2005) Gating reaction mechanisms for NMDA receptor channels. J. Neurosci. 25, 7914–7923
58. Talukder I., Wollmuth L. P. (2011) Local constraints in either the GluN1 or GluN2 subunit equally impair NMDA receptor pore opening. J. Gen Physiol. 138, 179–194

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