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A Novel Alcohol-Sensitive Position in the *N*-Methyl-D-Aspartate Receptor GluN2A Subunit M3 Domain Regulates Agonist Affinity and Ion Channel Gating

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ABSTRACT

Abundant evidence supports a role for *N*-methyl-D-aspartate (NMDA) receptor inhibition in the behavioral actions of ethanol, but the underlying molecular mechanisms have not been fully elucidated. We recently found that clusters of five positions in the third and fourth membrane-associated domains (M3 and M4) at the intersubunit interfaces form putative sites of alcohol action. In the present study, we found that one of these positions, NMDA receptor subunit, GluN2A(F636), can strongly regulate ethanol sensitivity, glutamate potency, and apparent desensitization: ethanol IC_{50} values, peak (I_p) and steady-state (I_{ss}) glutamate EC_{50} values, and steady-state to peak current ratio (I_{ss}:I_p) values differed significantly among the mutants tested. Changes in glutamate affinity among the various mutants were not attributable to agonist trapping due to desensitization, as glutamate peak EC_{50} values were correlated with values of both steady-state EC_{50} and I_{ss}:I_p. The mean open times determined in selected mutants could be altered up to 4-fold but did not account for the changes in ethanol sensitivity. Ethanol sensitivity was significantly correlated with glutamate EC_{50} and I_{ss}:I_p values, but the changes in ethanol IC_{50} among mutants at this position do not appear to be secondary to changes in ion channel kinetics. Substitution of the isomeric amino acids leucine and isoleucine had markedly different effects on ethanol sensitivity, agonist potency, and desensitization, which is consistent with a stringent structural requirement for ion channel modulation by the side chain at this position. Our results indicate that GluN2A(F636) plays an important role in both channel function and ethanol inhibition in NMDA receptors.

Introduction

Ethanol is among the most widely abused drugs in the world. A growing body of research shows that ethanol influences brain function through modulating the actions of multiple neurotransmitters, including glutamate and GABA (Valenzuela, 1997; Vengeliene et al., 2008), with actions on both neurotransmitter release and postsynaptic neurotransmitter-gated ion channels. Of the glutamate-gated ion channels, the *N*-methyl-D-aspartate (NMDA) receptor is considered to be particularly important as a target of ethanol (Woodward, 2000; Peoples, 2002; Krystal et al., 2003). Ethanol inhibits NMDA receptor gating rather than agonist binding (Gothert and Fink, 1989; Gonzales and Woodward, 1990; Snell et al., 1993; Peoples et al., 1997) by decreasing the channel opening frequency and the mean open time (Lima-Landman and Albuquerque, 1989; Wright et al., 1996). Studies designed to identify the NMDA receptor region containing the site of alcohol action found that the NMDA receptor C-terminal domain regulates ethanol sensitivity (Alvestad et al., 2003; Xu et al., 2011; Trepanier et al., 2012); however, this domain does not contain the primary site of alcohol action because truncation of the C-terminal domain does not abolish ethanol inhibition (Anders et al., 2000; Peoples and Stewart, 2000). Taken together with studies demonstrating the role of membrane-associated domains in NMDA receptor gating (Kohda et al., 2000; Jones et al., 2002; Sobolevsky et al., 2002a), these studies suggested that sites of ethanol action may be located in one or more of the membrane-associated (M) domains, as appears to be the case for alcohol and anesthetic sites on $GABA_A$ and glycine receptors (Mihic et al., 1997; Lobo et al., 2008; McCracken et al., 2010).

Reports from our laboratory and others have identified a small number of amino acid residues located in the M3 and M4 domains of NMDA receptor GluN1 and GluN2 subunits that influence ethanol sensitivity and channel function (Ronald et al., 2001; Ren et al., 2003b, 2007; Honse et al., 2004; Smothers and Woodward, 2006; Xu et al., 2012). The determinants of ethanol sensitivity and receptor function at each of these positions differ markedly from each other. Recently, we found four pairs of positions at the intersubunit interface in the M3 and M4 domains of the GluN1 and GluN2A subunits, including three positions identified previously (Ronald et al., 2001; Ren et al., 2003b, 2007), that interact with each other to regulate both ethanol sensitivity and measures of receptor function such as agonist deactivation and

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ANOVA, analysis of variance; APV, *dl*-2-amino-5-phosphonovaleric acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; GluN, NMDA receptor subunit; M, membrane-associated domain; NMDA, *N*-methyl-D-aspartate.

desensitization (Ren et al., 2012). In that study, we identified five positions in the GluN1 and GluN2A subunits that had not been previously shown to regulate ethanol sensitivity. Substitution mutations at a few of these individual positions, such as GluN2A(L824), had no effect on ethanol sensitivity by themselves (Honse et al., 2004) but influenced ethanol action only in concert with mutations at their paired position (Ren et al., 2012). In contrast, we found that tryptophan substitution at F636 in the M3 domain of GluN2A (Fig. 1) showed the lowest ethanol sensitivity among identified mutants in the GluN2A subunit (Ren et al., 2012), suggesting a greater relative importance of this position in the regulation of NMDA receptor alcohol sensitivity. We therefore investigated the role of this position in the regulation of NMDA receptor ethanol sensitivity and NMDA receptor function.

Materials and Methods

Ethanol (95%, prepared from grain) was obtained from Aaper Alcohol & Chemical Co. (Shelbyville, KY), and all other drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Site-Directed Mutagenesis, Cell Culture, and Transfection. Site-directed mutagenesis in plasmids containing GluN2A subunit cDNA was performed using the QuikChange II kit (Stratagene, La Jolla, CA), and all mutants were verified by double-strand DNA sequencing. Human embryonic kidney 293 cells were cultured to 70–95% confluence in minimum essential medium containing 10% heat-inactivated fetal bovine serum, Earle's salts, nonessential amino acids, sodium pyruvate, and L-glutamine at 37°C in 5% CO_2. Cells were plated in 35-mm poly-D-lysine–coated dishes and transfected with cDNA for the GluN1-1a and wild-type or mutant GluN2A subunits and green fluorescent protein (pGreen Lantern; Invitrogen, Carlsbad, CA) using a calcium phosphate transfection kit (Invitrogen). The cDNA ratio was typically 2:2:1 for GluN1:GluN2A:green fluorescent protein, respectively, but was adjusted to as low as 0.1:0.1:2.5 for GluN2A mutants that yielded very large currents. The NMDA receptor antagonists *dl*-2-amino-5-phosphonovaleric acid (APV), 200 μM, and ketamine, 100 μM, were added to the culture medium of each dish to protect the cells from receptor-mediated excitotoxicity after transfection. Recordings were made within 48 hours after transfection. The cells were extensively washed before use in the experiments.

Electrophysiologic Recording. Whole-cell recording was performed at room temperature using an Axopatch 1D or Axopatch 200B (Molecular Devices, Sunnyvale, CA) amplifier. In whole-cell recordings, patch-pipettes with open tip resistances of 1–8 MΩ were used. Series resistances of 2–7 MΩ were compensated by 80%. In single-channel experiments, pipettes had open tip resistances of 7–15 MΩ and were coated with DuPont elastomer R6101 (DuPont Performance Polymers, Wilmington, DE) to reduce capacitative noise. Cells were voltage-clamped at −50 mV and were superfused in an external recording solution containing (in mM) 150 NaCl, 5 KCl, 0.2 $CaCl_2$, 10 HEPES, 10 glucose, and 10 sucrose (pH 7.4).

Glycine (50 μM) was present in the external solution. Low calcium was used to minimize calcium-dependent inactivation (Medina et al., 1995; Kyrozis et al., 1996); in lifted-cell and single-channel experiments, the external solution also contained 10 μM EDTA to minimize Zn^{2+}-dependent desensitization (Erreger and Traynelis, 2005). The intracellular recording solution contained (in mM) 140 CsCl, 2 Mg_4ATP, 10 BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 10 HEPES (pH 7.2). Solutions of agonists and ethanol were applied to cells using a stepper motor-driven solution exchange apparatus (Warner Instruments, Hamden, CT) and 600 μm i.d. square glass tubing.

In the glutamate concentration-response experiments, cells were lifted off the surface of the dish to increase the speed of the solution exchange; under these conditions, 10–90% rise times for solution exchange are ~1.5 milliseconds. Concentration-response data were filtered at 2 kHz (8-pole Bessel) and acquired at 5 kHz on a computer using a DigiData interface and pClamp software (Molecular Devices). Single-channel data were acquired at 50 kHz and digitally filtered at 5 kHz (8-pole Bessel).

Data Analysis. In concentration-response experiments, IC_{50} or EC_{50} and n (slope factor) were calculated using the equation $y = E_{max}/1 + (IC_{50}$ or $EC_{50}/x)^n$, where y is the measured current amplitude, x is concentration, n is the slope factor, and E_{max} is the maximal current amplitude. Statistically significant differences among the concentration-response curves were determined by comparing log transformed EC_{50} or IC_{50} values from fits to the data obtained from individual cells using one-way analysis of variance (ANOVA) followed by the Dunnett test. Linear relations of mean values of log EC_{50}, log IC_{50}, or maximal steady-state to peak current ratio (I_{ss}:I_p) for the various mutants were made using linear regression analysis. All values are reported as mean ± S.E.

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/Molecular Devices). In most cells, deactivation was best fitted using a biexponential 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. Time constants of desensitization were determined similarly by fitting a monoexponential or biexponential function to the decay of current after the peak in the presence of a 10-second application of glutamate; weighted or monoexponential time constant values are reported.

Analysis of data from single-channel recordings was performed as described previously elsewhere (Ren et al., 2007). Data records were idealized using the segmentation K-means algorithm in the QUB software suite (http://www.qub.buffalo.edu/index.html) (Qin, 2004), open dwell time histograms were fitted with multiple exponential components using Clampfit (Molecular Devices), and mean open times were obtained from the proportionally weighted averages of the individual components. Single-channel conductances were determined by fitting a gaussian function to all-points amplitude histograms. Data were obtained from 3–22 patches for each GluN2A receptor mutant tested.

Results

Effects of Mutations at GluN2A(F636) on NMDA Receptor Ethanol Sensitivity. Previous studies have identified putative sites of ethanol action in the NMDA receptor membrane-associated domains (Peoples and Stewart, 2000; Ronald et al., 2001; Ren et al., 2003b, 2007; Honse et al., 2004; Smothers and Woodward, 2006). Figure 2B shows the percentage of inhibition by 100 mM ethanol in tryptophan substitution mutants from F636 to I640 in M3 of the GluN2A subunit. As can be seen, inhibition by 100 mM ethanol in GluN2A(F636W) was 10.8%, which was the lowest among the mutants. To test the role of this position in detail, we constructed a panel of substitution mutants at this position and tested their function using whole-cell recording. Except for the aspartate and arginine substitution mutants, which when coexpressed with GluN1 resulted in little or no measurable current, all other mutations tested at this position yielded functional receptors. Concentration-response experiments for ethanol inhibition revealed that all functional mutants were inhibited by ethanol in a concentration-dependent manner, as shown in Fig. 3. Substitution at GluN2A(F636) significantly altered ethanol IC_{50} values (ANOVA; $P < 0.0001$). Values of ethanol IC_{50} in the substitution mutants ranged

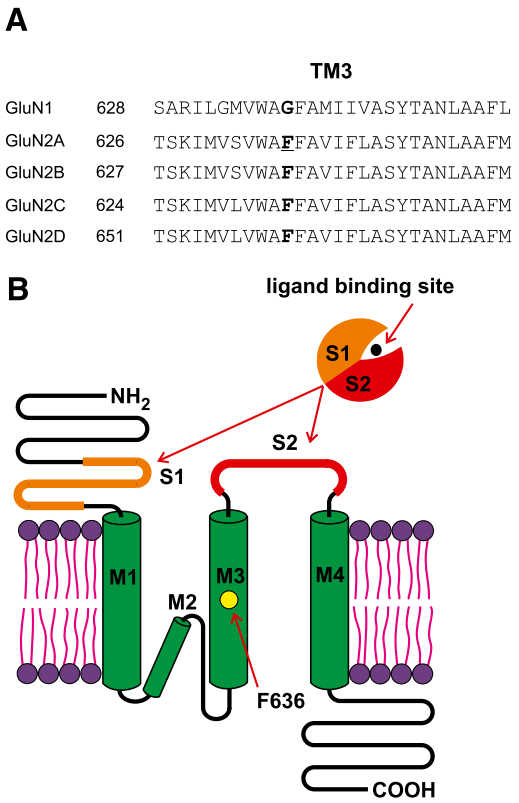

Fig. 1. Location of GluN2A(F636). (A) Partial sequences of the M3 domains in GluN1 and GluN2 subunits. F636 in M3 of the GluN2A subunit is underlined, and the cognate residues of F636 in GluN1 and GluN2B-D subunits are indicated in bold. (B) Topologic structure of the NMDA receptor subunit. The presumed location of F636 is noted by a yellow dot.

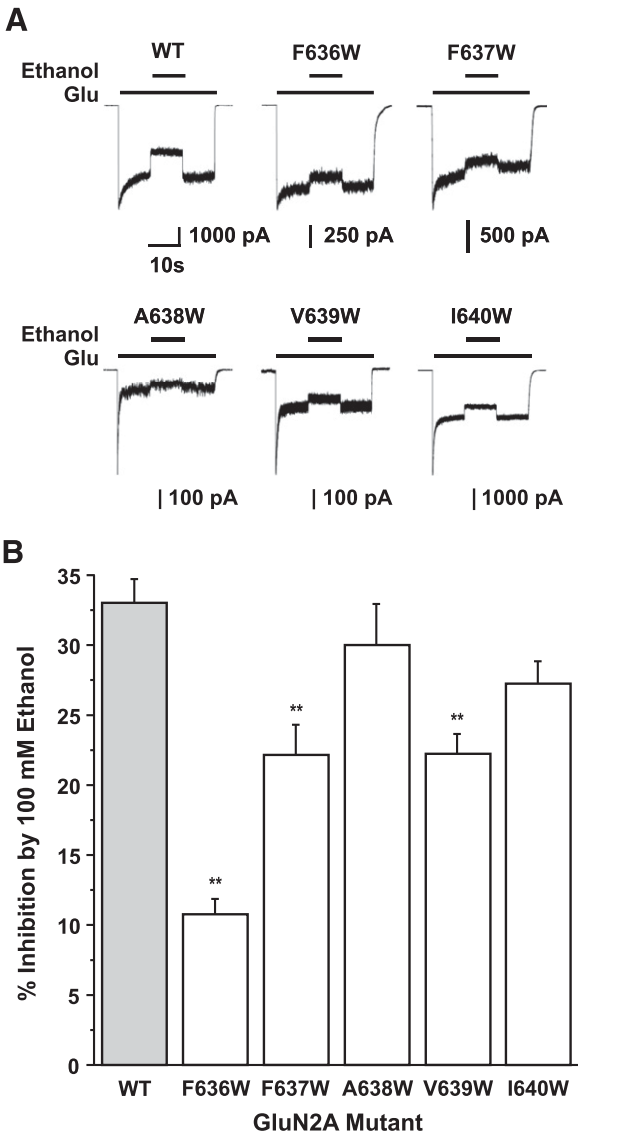

Fig. 2. Comparison of ethanol sensitivity among tryptophan substitution mutations at positions from F636 to I640 in M3 of the GluN2A subunit. (A) Traces are currents activated by 10 μM glutamate and 50 μM glycine and their inhibition by 100 mM ethanol in cells expressing GluN1 and wild-type (WT) GluN2A subunits or GluN2A subunits containing tryptophan substitution mutations at positions from F636 to I640. One-letter amino acid codes are used. (B) Bar graph shows the average percentage of inhibition by 100 mM ethanol of glutamate-activated current in cells expressing GluN1 and WT GluN2A subunits or GluN2A subunits containing tryptophan substitution mutations at positions from F636 to I640. Asterisks indicate values that are statistically significantly different from the WT GluN1/GluN2A subunit value (**$P < 0.01$; one-way ANOVA and Dunnett's test). Results are mean ± S.E. of 5–12 cells. Values for WT and GluN2A mutants F636W and F637W are from Ren et al. (2012).

from 191 to 451 mM, the values for the smallest substituent, glycine, and the largest substituent, tryptophan, respectively. None of the mutants exhibited increased ethanol sensitivity compared with the wild-type receptor. Interestingly, isoleucine substitution statistically significantly decreased ethanol sensitivity (IC_{50} value: 438 ± 13.6 mM; ANOVA and Dunnett's test; $P < 0.01$), whereas substitution with the isomeric amino acid leucine did not (IC_{50} value: 212 ± 6.14 mM; ANOVA and Dunnett's test; $P > 0.05$).

Effects of Mutations at GluN2A(F636) on NMDA Receptor Function. We and others have previously shown that alcohol-sensitive positions in M3 and M4 can regulate NMDA receptor function, including agonist potency, desensitization, and ion channel gating (Ronald et al., 2001; Ren et al., 2003b, 2007; Honse et al., 2004; Smothers and Woodward, 2006). To determine whether mutations at GluN2A(F636) also influence NMDA receptor characteristics, we compared EC_{50} values for glutamate activation of peak and steady-state currents among the mutants at this position using lifted cells and a fast solution exchange apparatus (Fig. 4; Table 1). Highly significant differences were obtained among the EC_{50} values for glutamate peak (ANOVA; $P < 0.0001$; Fig. 5A) and steady-state (ANOVA; $P < 0.0001$; Fig. 5B) currents as well as the steady-state to peak current ratio (I_{ss}:I_p; ANOVA; $P < 0.0001$; Fig. 5C). A majority of the mutants exhibited pronounced decreases in glutamate EC_{50} values (ANOVA; $P < 0.05$ or $P < 0.001$; Fig. 5, A and B), and the steady-state to peak current ratios were statistically significantly increased in all mutants, with the exception of the glycine and leucine substitutions (ANOVA; $P < 0.05$ or $P < 0.001$; Fig. 5C).

In a previous study, we had observed that mutations at a single position in the GluN2 AM4 domain, M823, or dual mutations at M823 and F637 in the M3 domain, altered glutamate EC_{50} values for steady-state current in a manner that was linked to maximal I_{ss}:I_p values. In the present study, we found that although there was a strong correlation between peak and steady-state glutamate EC_{50} values (ANOVA; $P < 0.0001$; Fig. 5D), the maximal I_{ss}:I_p values

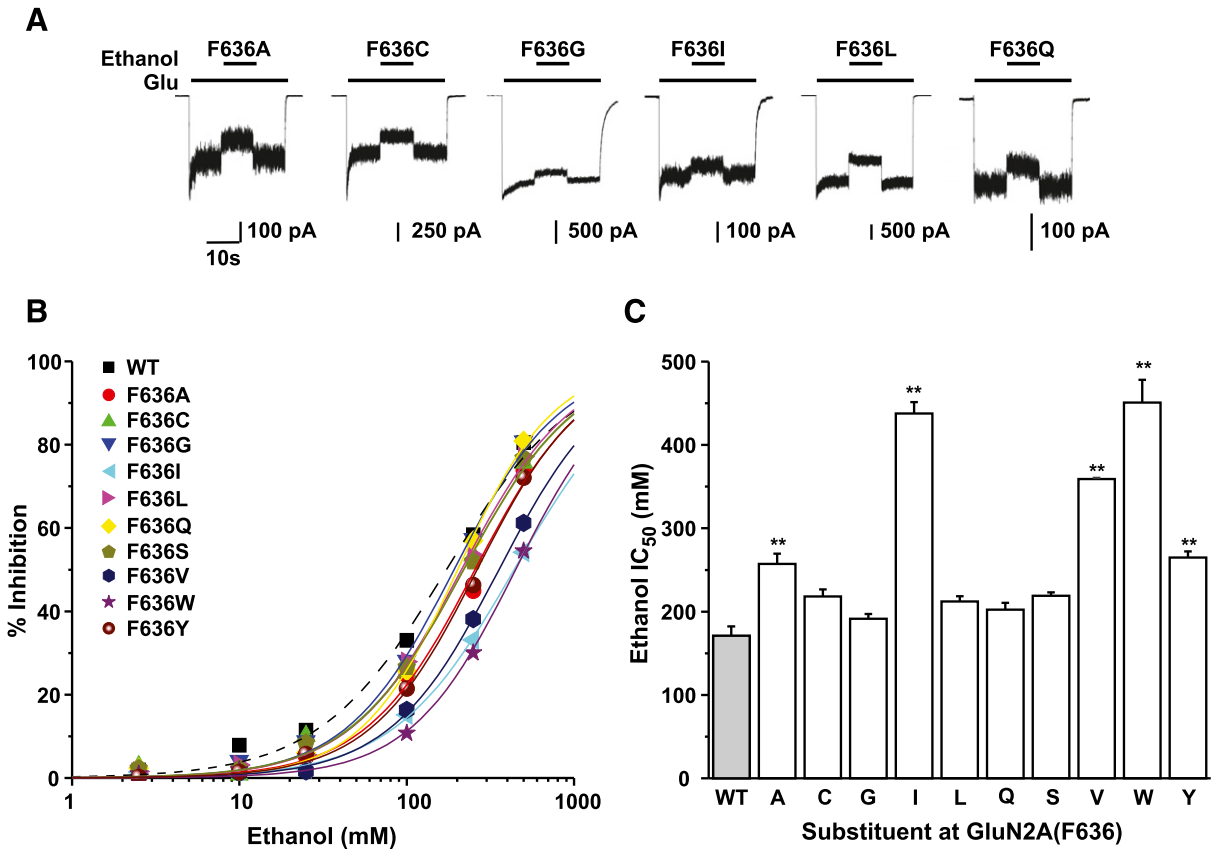

Fig. 3. Mutations at GluN2A(F636) can alter NMDA receptor ethanol sensitivity. (A) Traces are currents activated by 10 μM glutamate and 50 μM glycine and their inhibition by 100 mM ethanol in cells expressing GluN1 and wild-type (WT) GluN2A subunits or GluN2A subunits containing various substitution mutations at F636. (B) The concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressed various substitution mutations at GluN2A(F636). Data are the mean ± S.E. of 5–12 cells, and error bars are not shown for clarity. The curves shown are the best fits to the equation given in *Materials and Methods*. The dashed line shows the fit for the WT GluN2A subunit. One-letter amino acid codes are used. (C) Bar graph shows the average IC_{50} values for ethanol inhibition of glutamate-activated current in cells expressing GluN1 and WT GluN2A subunits or GluN2A subunits containing various mutations at GluN2A(F636). Asterisks indicate IC_{50} values that are statistically significantly different from that for the WT GluN1/GluN2A subunit (**$P < 0.01$; one-way ANOVA and Dunnett's test). Results are the mean ± S.E. of 5–12 cells. Values for WT and GluN2A mutants F636W and F637W are from Ren et al. (2012).

among mutants at F636 were correlated with EC_{50} values of glutamate activated peak current (ANOVA; $P < 0.05$; Fig. 6) but not with the values for steady-state current (ANOVA; $P > 0.05$).

We also obtained evidence that substitutions at F636 can alter receptor kinetics, including apparent desensitization, deactivation after removal of agonist, and the mean open time of the ion channel. In whole-cell recordings from lifted cells, apparent desensitization of glutamate-activated current and deactivation of current after rapid removal of glutamate could both be altered by mutations at F636 (Table 2).

Because the presence of magnesium has been reported to alter ethanol inhibition of NMDA receptors (Jin et al., 2008), we tested magnesium sensitivity in the least ethanol-sensitive mutant, GluN2A(F636W). In receptors containing the GluN2A (F636W) subunit, the IC_{50} value for magnesium inhibition was not altered compared with that for receptors bearing the wild-type GluN2A subunit (87.9 ± 7.45 versus 97.1 ± 7.18 μM, respectively; *t* test, $P > 0.05$). In single-channel recordings from outside-out patches, the mean open time was not significantly changed by substitution of alanine or isoleucine at this position, but was significantly decreased by substitution of leucine and prolonged by substitution of tyrosine or tryptophan (Fig. 7; Table 3). Single-channel conductance was not significantly altered in the mutants tested, with the exception of the leucine substitution mutant, in which it was decreased (Table 3). In this mutant, the very brief open times of most of the events appeared to prevent them from reaching full amplitude.

GluN2A(F636) Mutant Ethanol Sensitivity and NMDA Receptor Function. To determine whether changes in ethanol sensitivity, agonist potency, and apparent desensitization among the mutants at F636 depended on the physical-chemical properties of the substituents at this position, we tested whether values of glutamate EC_{50} (Fig. 8, A–C), ethanol IC_{50} (Fig. 8, D–F), and I_{ss}:I_p ratios (not shown) were linearly related to amino acid polarity, hydrophilicity, or molecular volume. No statistically significant linear relations were obtained among the any of the measures tested. However, we noticed marked differences in mutants containing the isomeric substituents leucine and isoleucine. Substitution of isoleucine at F636 not only statistically significantly altered ethanol sensitivity, as mentioned previously (ANOVA; $P < 0.01$; Fig. 3), but also statistically significantly decreased the glutamate peak (ANOVA; $P < 0.001$) and steady-state (ANOVA; $P < 0.001$) EC_{50} and apparent desensitization (ANOVA; $P < 0.001$); the isomeric amino acid, leucine, did not influence any of these values (Fig. 5).

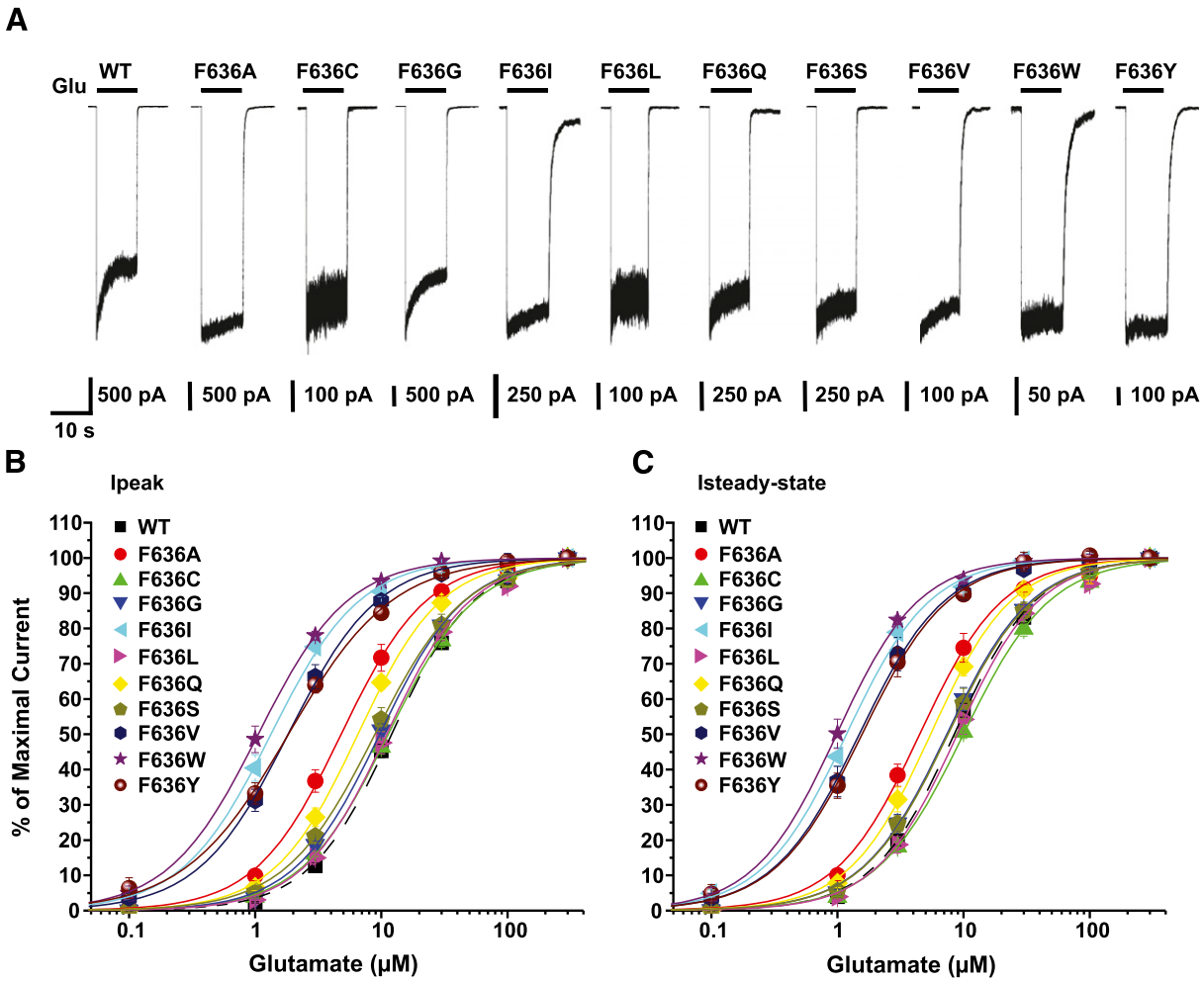

Fig. 4. Mutations at GluN2A(F636) can alter glutamate EC_{50}. (A) Traces are currents activated by 300 μM glutamate in the presence of 50 μM glycine in lifted cells expressing wild-type (WT) GluN1/GluN2A or various GluN1/GluN2A(F636) mutant subunits. (B and C) Concentration-response curves for glutamate-activated peak (B) and steady-state (C) currents in lifted cells expressing wild-type GluN1/GluN2A or GluN1/GluN2A(F636) mutant subunits. Data points are the mean ± S.E. of 5–12 cells, the error bars not visible were smaller than the size of the symbols, and the curves shown are the best fits to the equation given in the *Materials and Methods*. The dashed line shows the fit for the wild-type GluN2A subunit.

Plotting the ethanol IC_{50} values against glutamate EC_{50} or I_{ss}:I_p values revealed that the ethanol sensitivity of the mutants was statistically significantly negatively dependent on both the glutamate peak ($R^2 = 0.86$; $P < 0.0001$) and the steady-state EC_{50} values ($R^2 = 0.85$, $P < 0.0001$; Fig. 9), and was also statistically significantly correlated with the maximal steady-state to peak current ratio ($R^2 = 0.45$; $P < 0.05$; Fig. 9).

TABLE 1
Current amplitudes of GluN2A(F636) mutants

Substituent	I_p	I_{ss}	n
	pA		
WT	3550 ± 995	2070 ± 538	13
A	2190 ± 746	2090 ± 698	5
C	2450 ± 922	1940 ± 621	6
G	5190 ± 1470	3500 ± 931	6
I	996 ± 399	919 ± 363	6
L	1940 ± 878	1400 ± 617	5
Q	1900 ± 825	1460 ± 572	6
S	2400 ± 913	1950 ± 654	5
V	2940 ± 1460	2120 ± 923	7
W	924 ± 262	845 ± 230	5
Y	1580 ± 914	1400 ± 783	5

WT, wild-type.

Discussion

Substitution mutants at GluN2A(F636) could significantly alter ethanol sensitivity, mean open time, apparent desensitization, and apparent agonist affinity. Although mutations at other alcohol-sensitive positions in the GluN2A subunit can alter glutamate affinity, the underlying mechanism at F636 appears to differ from those previously reported. Increased the steady-state glutamate apparent affinity in mutants at M823 in the M4 domain was highly correlated with apparent desensitization and was attributable to increased dwell times in one or more desensitized states, resulting in trapping of agonist (Ren et al., 2003a). However, for mutants at F636 the apparent desensitization had an inverse relationship with the glutamate peak EC_{50} and a parallel trend with the glutamate steady-state EC_{50}. Taken together with our observation of a strong correlation between peak and steady-state EC_{50} values, these results are not

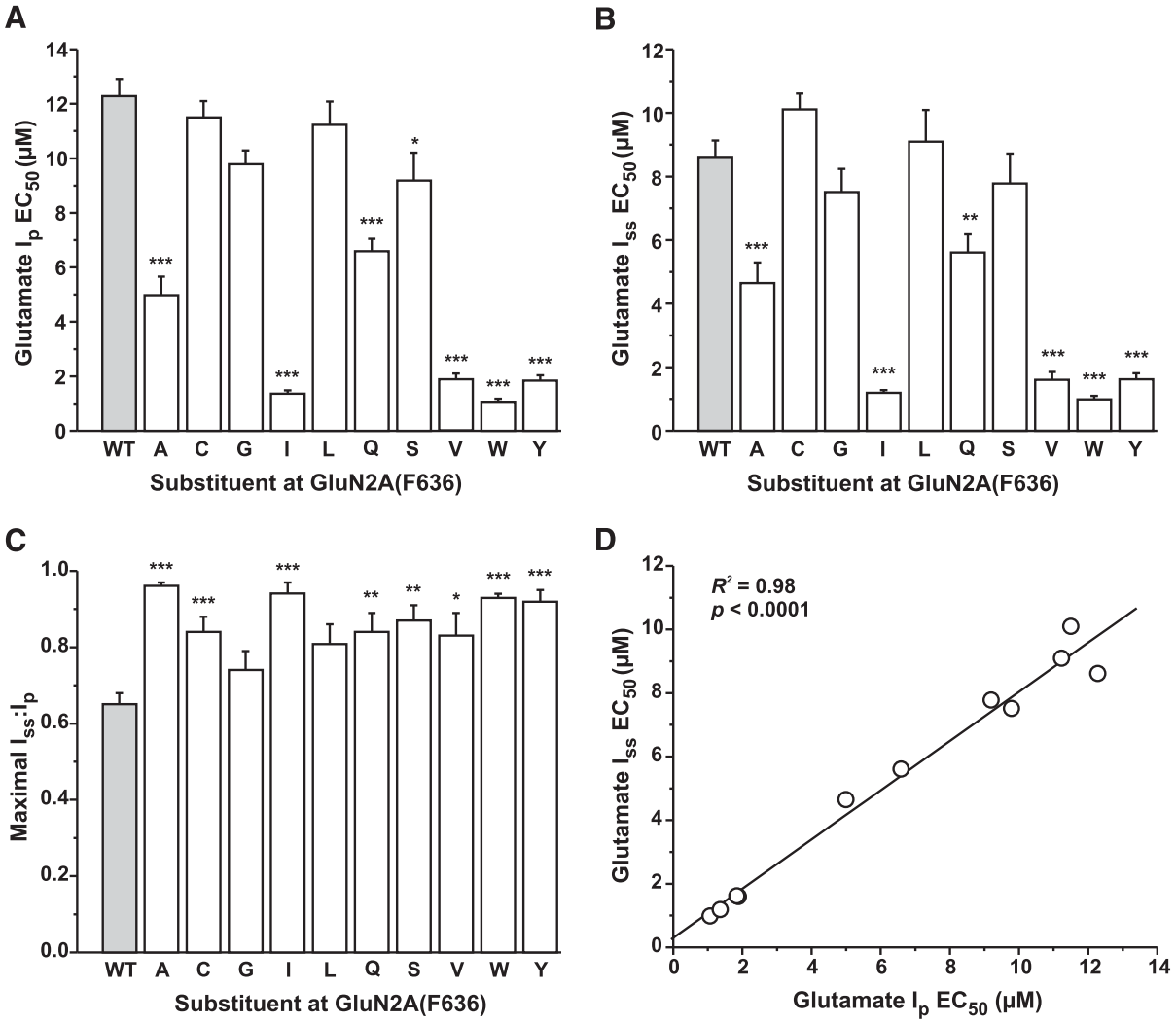

Fig. 5. Mutations at GluN2A(F636) can alter glutamate potency and desensitization. (A and B) Bar graphs show the average EC_{50} values for glutamate activated peak (A) and steady-state (B) currents in lifted cells expressing GluN1 and wild-type (WT) GluN2A subunits or GluN2A subunits containing various mutations at F636. Asterisks indicate EC_{50} values that are statistically significantly different from the value for WT GluN1/GluN2A subunits (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; one-way ANOVA and Dunnett's test). Results are the mean ± S.E. of 5–12 cells. (C) Bar graph shows the average values of maximal steady-state to peak current ratio (I_{ss}:I_p) in lifted cells coexpressing GluN1 and WT GluN2A subunits or GluN2A subunits containing various mutations at F636. Currents were activated by 300 μM glutamate in the presence of 50 μM glycine. Asterisks indicate values that were statistically significantly different from the value for WT GluN1a/GluN2A subunits (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; one-way ANOVA and Dunnett's test). Results are the mean ± S.E. of 5–12 cells. (D) The graph plots values of glutamate log EC_{50} for steady-state current versus values of glutamate log EC_{50} for peak current in the series of mutants. Glutamate EC_{50} values for peak and steady-state current were statistically significantly correlated ($R^2 = 0.98$; $P < 0.0001$). The line shown is the least-squares fit to the data.

consistent with a mechanism involving agonist trapping due to increased desensitization (Ren et al., 2003a).

Similarly, our results differ from those observed for mutants at F637. Of the substitution mutations tested at F637, desensitization was markedly increased in two mutants and was not significantly altered in the remainder (Ren et al., 2007), whereas desensitization was decreased in 8 of 10 substitution mutants at F636 in our study. Furthermore, the mean open time was decreased from 2- to 7-fold in all mutants tested at F637, while our results indicate that in five mutants tested to date at F636 the mean open time was unchanged in two mutants, decreased nearly 5-fold in one mutant, and prolonged 3- to 4-fold in the remaining two mutants. Thus, although F636 and F637 are adjacent positions and both modulate NMDA receptor ethanol sensitivity, their actions on ion channel kinetics differ considerably.

The changes we observed in glutamate EC_{50} among mutants at F636 most likely stem from modifications in channel gating because F636 is within the M3 domain at a considerable distance from the agonist binding site (Buck et al., 2000; Sobolevsky et al., 2007, 2009) and because of the role of the M3 segment in NMDA receptor ion channel gating (Jones et al., 2002; Sobolevsky et al., 2002b; Low et al., 2003; Yuan et al., 2005). However, the explanation for the changes in affinity is not clear and may differ among mutants at this position. For example, in the isoleucine and tryptophan mutants, we observed prolonged glutamate deactivation time constants that are consistent with the decreased glutamate IC_{50} values in these mutants, but the mean open time was markedly increased in the tryptophan mutant and unchanged in the isoleucine mutant. Because the agonist cannot dissociate from its binding site when the channel is in the open state, the

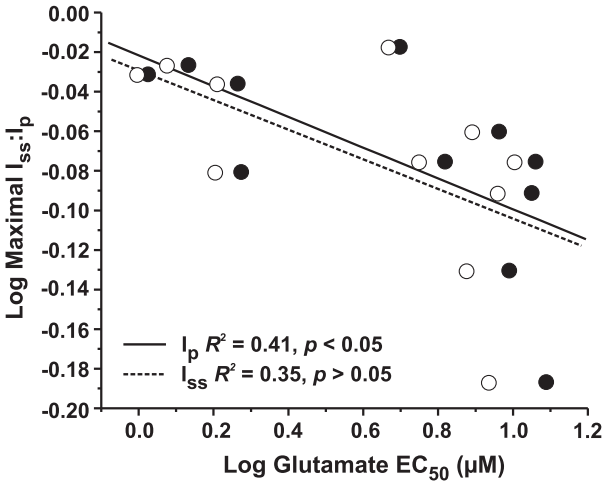

Fig. 6. Relationship between glutamate EC_{50} and desensitization. The graph plots the log I_{ss}:I_p versus steady-state (○) and peak (●) log EC_{50} values for glutamate-activated current in various GluN2A(F636) mutant subunits. Log maximal I_{ss}:I_p for glutamate was correlated with peak glutamate log EC_{50} (R^2 = −0.41; $P < 0.05$) but not with steady-state glutamate log EC_{50} (R^2 = 0.35; $P > 0.05$). The lines shown are the least squares fits to the data.

prolonged channel open time in the tryptophan mutant could account at least in part for the increased duration of agonist occupancy, increasing agonist affinity. However, this explanation cannot account for the increase in apparent agonist affinity in the isoleucine mutant, in which the mean open time was not significantly altered. Resolution of this question will likely require detailed kinetic modeling to obtain rate constants for all of the gating transitions involved. What is clear at present is that the regulation of ion channel gating at position 636 is exquisitely sensitive to the structure of the side chain, as evidenced by the marked differences in the effects of the isomeric substituents leucine and isoleucine on agonist affinity, apparent desensitization, and mean open time.

Substitution mutations at GluN2A(F636) could also significantly alter ethanol sensitivity. Ethanol inhibition of the NMDA receptor is not determined by a single position in the membrane-associated domains, but rather the sites of ethanol action on the NMDA receptor appear to be composed of several interacting positions located in the M domains (Ren et al., 2012), each of which possess unique characteristics (Ronald et al., 2001; Ren et al., 2003b, 2007; Smothers and Woodward, 2006). For previously identified ethanol-sensitive positions in GluN2A, ethanol sensitivity was dependent on the molecular volume and hydrophilicity of the substituent at M823 (Ren et al., 2003b), was inversely dependent upon the molecular volume of the substituent at F637 (Ren et al., 2007), but was not correlated with any physicochemical measure of the substituent at A825 (Salous et al., 2009). In the GluN1 subunit, ethanol sensitivity was correlated with the molecular volume of the side chain at F639 (Smothers and Woodward, 2006), the

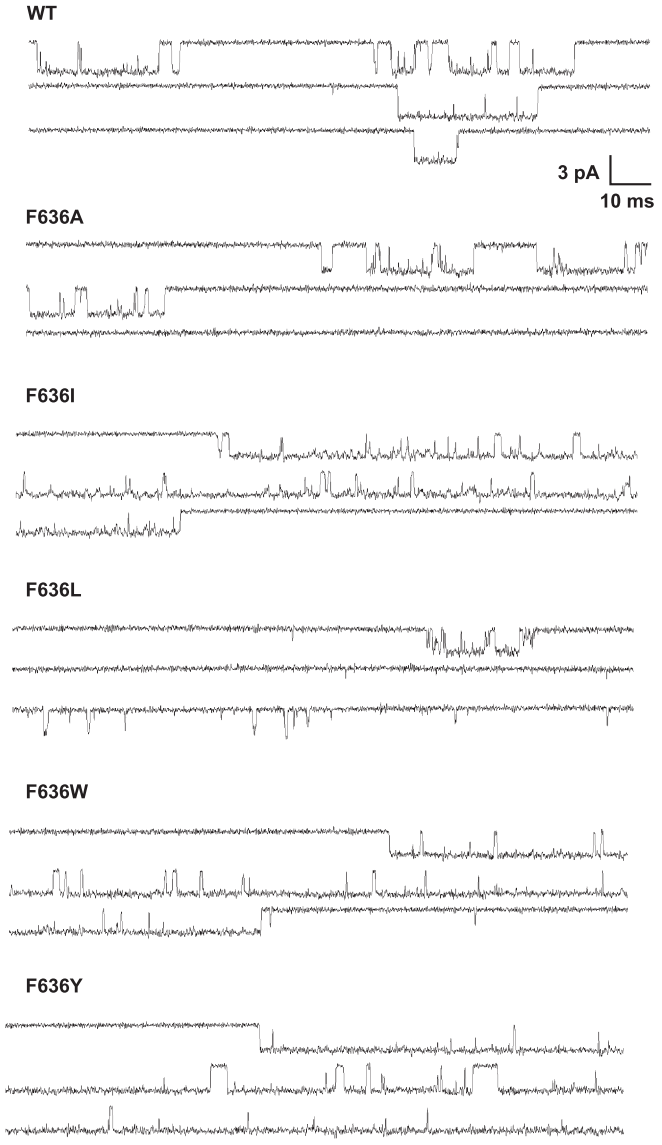

Fig. 7. Single-channel currents in outside-out patches from cells expressing wild-type (WT) and F636 mutant GluN2A subunits. Traces are currents activated by glutamate and 50 μM glycine in outside-out patches from cells expressing GluN1 and WT or F636 mutant GluN2A subunits. The glutamate concentration was 100 nM in the F636I and F636Y mutants, and was 1 μM for the WT and all other mutant subunits. Openings are downward; the scale bar in the first record applies to all traces.

TABLE 2
Kinetic measures in selected GluN2A(F636) mutants

Substituent	Deactivation τ	Desensitization τ	n
	ms		
WT	48.2 ± 3.92	2330 ± 257	13
I	1140 ± 224[a]	5250 ± 996	5
L	36.7 ± 3.01	2000 ± 682	5
W	960 ± 128[a]	8960 ± 2670[b]	5

WT, wild-type.
[a]$P < 0.001$ versus wild type (ANOVA and Dunnett's test).
[b]$P < 0.01$ versus wild type (ANOVA and Dunnett's test).

TABLE 3
Mean open times in GluN2A(F636) mutants

Substituent	Mean Open Time	Single-Channel Conductance	n
	ms	*pS*	
WT	3.38 ± 0.206	57.0 ± 1.58	22
A	3.09 ± 0.418	50.6 ± 2.18	5
I	4.61 ± 0.422	57.7 ± 2.17	5
L	0.780 ± 0.138[a]	39.0 ± 0.945[a]	5
W	13.8 ± 1.63[a]	63.2 ± 1.69	9
Y	12.4 ± 0.974[a]	62.0 ± 1.62	3

WT, wild-type.
[a]$P < 0.01$ (ANOVA and Dunnett's test).

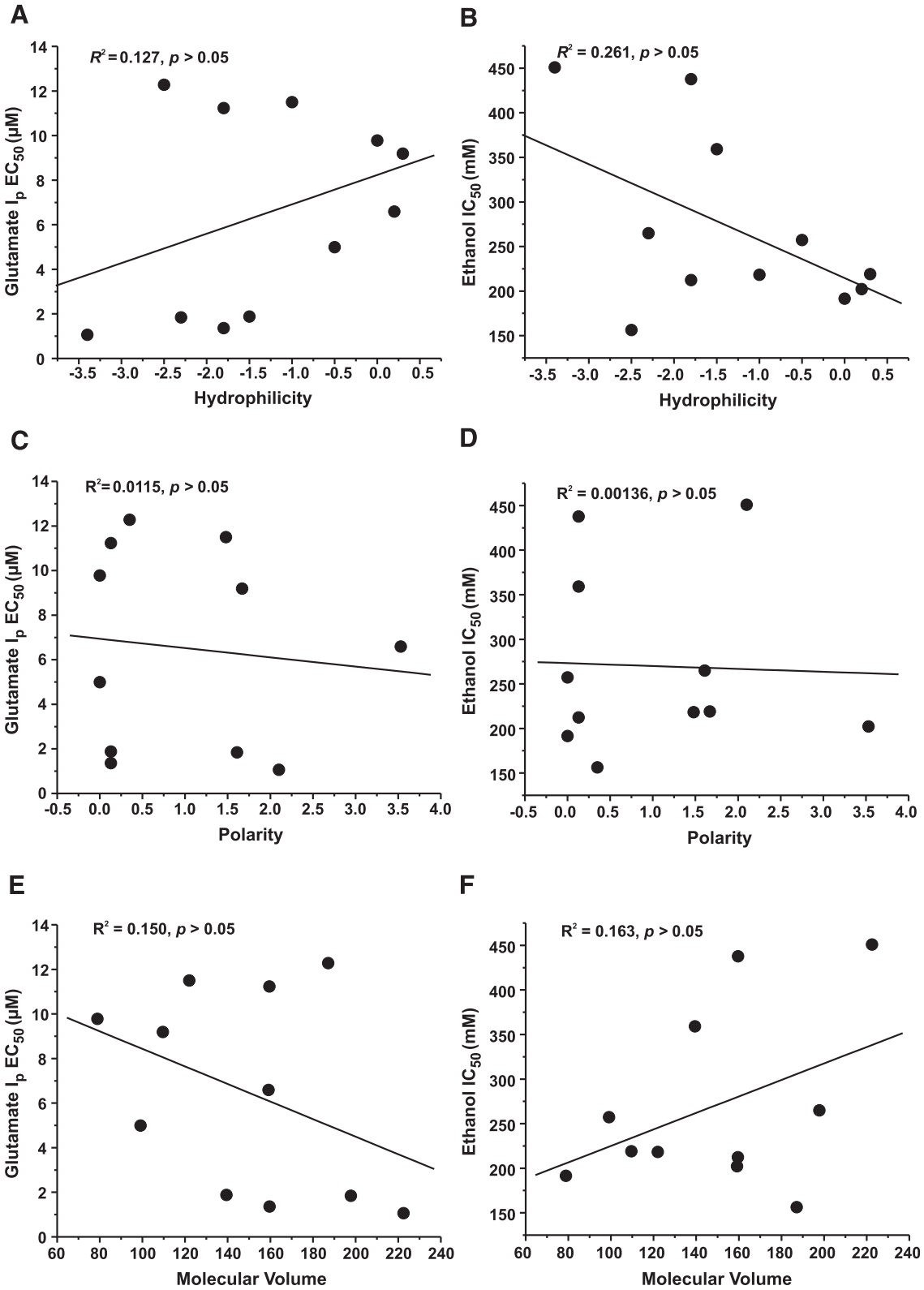

Fig. 8. Relation of NMDA receptor agonist potency and ethanol sensitivity with the physicochemical parameters of the substituent at GluN2A(F636). The graphs plot glutamate peak EC_{50} values (A, C, and E) or ethanol IC_{50} values (B, D, and F) versus hydrophilicity (A and B), polarity (C and D), and molecular volume (E and F) in various GluN2A(F636) mutant subunits. No statistically significant linear relations were obtained among any of the measures tested ($P > 0.05$).

cognate position to GluN2A(F637). In our present study, ethanol sensitivity was not correlated with any physicochemical parameter of the amino acid substituent at F636.

In all these studies, the finding that substitution mutations at a given position alter ethanol sensitivity indicates that the side chain at the position interacts with adjacent side chains,

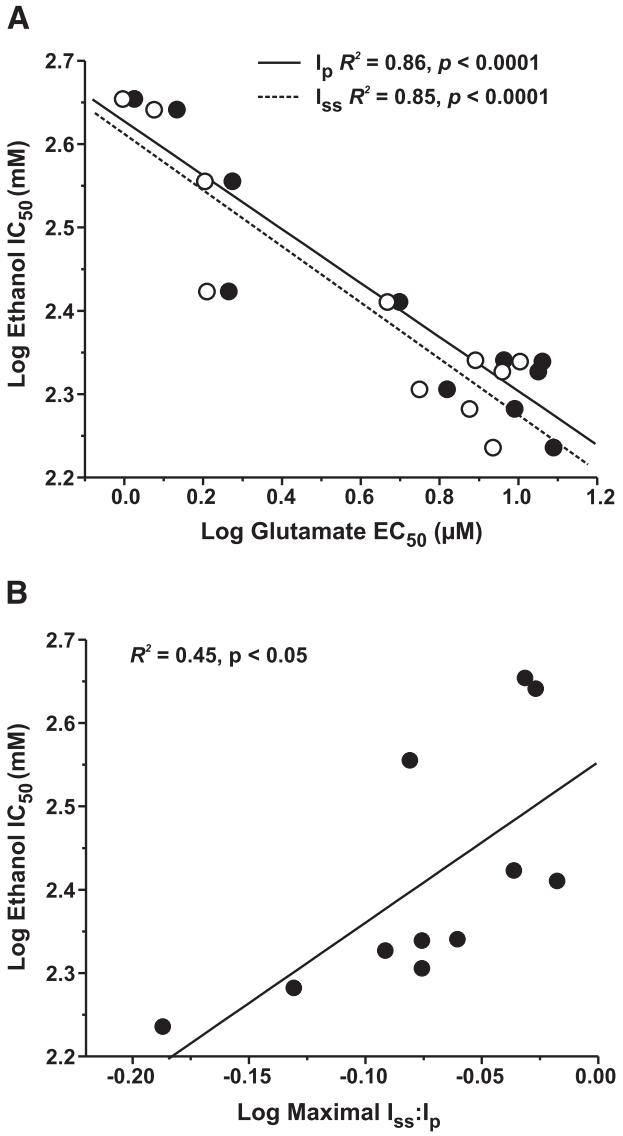

Fig. 9. Ethanol sensitivity of NMDA receptors containing GluN2A (F636) mutant subunits is related to glutamate apparent affinity and apparent desensitization. (A) The graph plots values of log ethanol IC_{50} in a series of mutants versus glutamate log EC_{50} for peak (●) and steady-state (○) current. Log ethanol IC_{50} was statistically significantly negatively correlated with glutamate EC_{50} for both peak (R^2 = 0.86; $P < 0.0001$; solid line) and steady-state (R^2 = 0.85; $P < 0.0001$; dashed line) current. The lines shown are the least-squares fits to the data. (B) The graph plots values of log ethanol IC_{50} versus maximal I_{ss}:I_p for glutamate-activated current in various GluN2A(F636) mutant subunits. Log ethanol IC_{50} was correlated with log maximal I_{ss}:I_p values (R^2= 0.45; $P < 0.05$). The lines shown are the least squares fits to the data.

and perhaps with the ethanol molecule as well, in a manner that alters receptor function. Positive correlations between substituent side chain volume and ethanol sensitivity are consistent with a model in which ethanol acts by occupying a critical volume, as has been shown in other alcohol-sensitive ligand-gated ion channels (Wick et al., 1998; Ye et al., 1998; Koltchine et al., 1999; Yamakura et al., 1999; Kash et al., 2003). The lack of such a correlation in our study may indicate that the side chain at 636 does not project into the interior of an alcohol binding site but rather forms part of the outer boundary of such a site (Ren et al., 2012). In this case, the side chain at 636 would regulate receptor function in a more complex manner depending on its interactions with other side chains in its environment. The striking difference in ethanol IC_{50} values observed between the mutants with the isomeric substituents leucine and isoleucine, which have virtually identical physicochemical characteristics, is consistent with this view.

The M3 domain is highly conserved in NMDA receptors (Yuan et al., 2005), which is consistent with its central role in ion channel gating. Residues from V633 to A638 in the GluN2 AM3 domain are conserved across all GluN1 and GluN2 subunits with the exception of a glycine residue at the position corresponding to F636 in GluN1. In our study, substitution of glycine at GluN2A(F636) did not alter ethanol sensitivity or ion channel function. Although this observation could help explain the presence of such disparate residues as glycine and phenylalanine in an otherwise highly conserved region of GluN1 and GluN2 subunits, the explanation for the lack of effect of glycine substitution at GluN2A(F636) is not clear at present. Substitutions such as alanine or valine at GluN2A (F636) strongly influenced ion channel function, and a subtle structural change of the side chain at this position from leucine to isoleucine profoundly altered ethanol inhibition and glutamate potency. Our observations may indicate that a phenylalanine at this position has a neutral effect on glutamate potency and ethanol sensitivity, equivalent to the absence of the side chain, whereas other hydrophobic substituents disrupt functioning. Further studies will be needed to understand how changes at GluN2A(F636) affect the structure of this region and its influence on ion channel function.

Ethanol inhibition of the NMDA receptor was inversely correlated with glutamate EC_{50} such that mutations at F636 that had higher agonist affinity were less sensitive to ethanol. A similar phenomenon was observed in mutations at F637 in the GluN2A subunit (Ren et al., 2007). Although it is possible that the changes in ethanol sensitivity we observed were secondary to changes in ion channel gating or agonist affinity, previous studies have not supported agonist affinity per se as a cause for the changes of ethanol sensitivity (Peoples et al., 1997; Ren et al., 2003b), and the significant correlation between ethanol sensitivity and glutamate EC_{50} among various mutants at F636 does not exclude the possibility that similar factors may influence both ethanol sensitivity and agonist affinity.

We also observed a significant correlation between ethanol sensitivity and apparent desensitization among mutants at F636. These results are in contrast to our earlier findings for mutants at GluN2A(M823), at which there is an inverse correlation between apparent desensitization and ethanol sensitivity (Ren et al., 2003b). Ethanol has been shown to influence desensitization in several ligand-gated ion channels, including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), 5-hydroxytryptamine (5-HT), and GABA receptors (Moykkynen et al., 2003, 2009; Dopico and Lovinger, 2009). It remains possible that ethanol inhibition may involve one or more components of desensitization, but the opposite effects of mutations at F636 and M823 on ethanol sensitivity and desensitization agree with previous findings (Peoples et al., 1997; Woodward, 2000) that suggest that ethanol inhibition of NMDA receptors does not involve changes in desensitization. Furthermore, as we previously reported for mutants at GluN2A (F637) (Ren et al., 2007), our study found that ethanol inhibition

in cells expressing the GluN2A(F636W) subunit did not differ when ethanol was applied during steady-state current and when it was preapplied for 10 seconds before measuring peak current (18.8 ± 1.18 versus 22.3% ± 0.955% inhibition by 250 mM ethanol, respectively; one-way ANOVA, $P > 0.05$). It appears most likely that the relationship between ethanol and desensitization we observed may be due to similar factors that modulate ethanol sensitivity and apparent desensitization in parallel.

Authorship Contributions

Participated in research design: Peoples, Ren.
Conducted experiments: Ren, Zhao, Wu.
Performed data analysis: Ren, Peoples, Zhao, Wu.
Wrote or contributed to the writing of the manuscript: Peoples, Ren.

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