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Intersubunit Interactions at Putative Sites of Ethanol Action in the M3 and M4 Domains of the NMDA Receptor GluN1 and GluN2B Subunits

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Intersubunit Interactions at Putative Sites of Ethanol Action in the M3 and M4 Domains of the NMDA Receptor GluN1 and GluN2b Subunits

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Abstract

Background and purpose: The *N*-methyl-D-aspartate (NMDA) receptor is an important target of alcohol action in the brain. Recent studies in this laboratory have demonstrated that alcohol-sensitive positions in the intersubunit interfaces of the M3 and M4 domains of GluN1 and GluN2A subunits interact with respect to ethanol sensitivity and receptor kinetics, and that alcohol-sensitive positions in the M domains of GluN2A and GluN2B subunits differ. In this study we tested for interactions among alcohol-sensitive positions at the M domain intersubunit interfaces in GluN1/GluN2B NMDA receptors.

Experimental approach: We used whole-cell patch-clamp recording in tsA201 cells expressing tryptophan substitution mutants at ethanol-sensitive

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

positions in the GluN1 and GluN2B NMDA receptor subunits to test for interactions among positions.
Key results: Six pairs of positions in GluN1/GluN2B significantly interacted to regulate ethanol inhibition: Gly638/Met824, Gly638/Leu825, Phe639/Leu825, Phe639/Gly826, Met818/Phe637 and Val820/Phe637. Tryptophan substitution at Met824 or Leu825 in GluN2B did not alter ethanol sensitivity, but interacted with positions in the GluN1 M3 domain to regulate ethanol action, whereas tryptophan substitution at Gly638, which is the cognate of an ethanol-sensitive position in GluN2A, did not alter ethanol sensitivity or interact with positions in GluN1. Two and three pairs of positions interacted to regulate glutamate steady-state and peak current EC_{50}, respectively, and one pair interacted with respect to macroscopic desensitization.
Conclusions: Despite highly-conserved M domain sequences and similar ethanol sensitivity in the GluN2A and GluN2B subunits, the manner in which these subunits interact with the GluN1 subunit to regulate ethanol sensitivity and receptor kinetics differs.

Keywords: glutamate receptor; alcohol; membrane-associated domains; electrophysiology; mutant

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; EtOH, Ethanol

Introduction

Alcohol abuse and alcoholism are behavioral disorders involving altered synaptic transmission in the CNS (Koob, 2003; Gass and Olive, 2008). The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate-gated ion channel, is among the most important target sites of alcohol in the brain (Woodward, 1999; Krystal *et al*., 2003; Peoples, 2003; Chandrasekar, 2013). At relevant concentrations, ethanol inhibits NMDA receptor-mediated ionic current via changes in channel mean open time and opening frequency (Lima-Landman and Albuquerque, 1989; Lovinger *et al*., 1989; Wright *et al*., 1996). Ethanol appears to inhibit NMDA receptors via low affinity interactions with a number of positions in the membrane-associated (M) domains that modulate ion channel gating (Ronald *et al.*, 2001; Ren *et al.*, 2003a, 2003b, 2007, 2012, 2013; Honse *et al.*, 2004; Smothers and Woodward, 2006; Zhao *et al.*, 2015), but the manner in which ethanol interacts with its molecular sites to modulate the activity of NMDA receptors is still incompletely understood.

Previous studies in this and other laboratories have identified a number of amino acid positions in NMDA receptor GluN1 and GluN2A

subunit membrane-associated (M) domains that influence both gating and alcohol sensitivity of the ion channel. Following the initial finding that a position in the M3 domain of the GluN1 subunit, Phe639, can regulate NMDA receptor ethanol sensitivity (Ronald *et al*., 2001), a number of studies from this laboratory in the GluN2A subunit found that the cognate position, Phe637, in the M3 domain, and three other positions in the M3 and M4 domains, Phe636, Met823, and Ala825, regulate NMDA receptor ethanol inhibition and ion channel kinetics (Ren *et al*., 2003a, 2003b, 2007, 2012, 2013; Honse *et al*., 2004). Previous studies have demonstrated that these and other positions in the GluN1 and GluN2A subunits can interact to regulate ethanol sensitivity and ion channel function (Smothers and Woodward, 2006; Ren *et al*., 2008, 2012; Xu *et al.*, 2015). The results of these studies, taken together with the solved structures of ionotropic glutamate receptors that show the identified alcohol-sensitive positions in the M3 domain of one subunit type closely apposed to those in the M4 domain of the other subunit type (Sobolevsky *et al.*, 2009; Karakas and Furukawa, 2014), predict the existence of four sites of alcohol action: two at the GluN1 M3/GluN2A M4 interfaces, and the other two at the GluN1 M4/GluN2A M3 interfaces (Ren *et al*., 2012).

Although the GluN2A subunit predominates in the mammalian brain, a number of studies suggest a major role for the GluN2B subunit in the action of alcohol on the brain (Boyce-Rustay and Holmes, 2005; Kash *et al*., 2008, 2009; Wills *et al.*, 2012), and consequently for the importance of the GluN2B subunit as a potential therapeutic target for the treatment of alcohol addiction (Chazot, 2004; Nagy, 2004; Gogas, 2006; Holmes *et al.*, 2013). At present, however, the understanding of the molecular mechanism of alcohol modulation of the GluN2B subunit is limited. A recent study from this laboratory reported that alcohol-sensitive positions at the M3-M4 intersubunit interfaces of GluN1/GluN2A NMDA receptors can interactively regulate both alcohol sensitivity and ion channel kinetics (Ren *et al*., 2012). Based on the high homology between the GluN2A and GluN2B subunit M domains (Ryan *et al*., 2013), we tested whether we could observe similar interactions among the cognate positions in GluN1/GluN2B NMDA receptors. In the present study, we report that multiple pairs of positions in the GluN1/GluN2B NMDA receptor interact to modulate ethanol inhibition and ion channel gating. Compared to previous

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

results obtained in the GluN1/GluN2A NMDA receptor, the interactions we observed among positions in the GluN1/GluN2B NMDA receptor differ with respect to both ethanol sensitivity and ion channel kinetics, which is consistent with previous observations of differences in alcohol-sensitive positions between the GluN2A and GluN2B subunits (Zhao *et al.*, 2015).

Methods

Site-directed mutagenesis, cell culture and transfection

Site-directed mutagenesis in plasmids containing GluN1 or GluN2B subunit cDNA was performed using the QuickChange II kit (Agilent Technologies, Santa Clara, CA, USA), and all mutations were verified by DNA sequencing. Transformed human embryonic kidney (tsA 201) cells were seeded in 35-mm poly-D-lysine coated dishes, and cultured in minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum to 70 - 95% confluence. Cells were transfected with cDNA for the GluN1-1a, GluN2B subunits and green fluorescent protein (pGreen Lantern; Invitrogen, Carlsbad, CA) at a 2:2:1 ratio using calcium phosphate transfection kit (Invitrogen). After transfection, 200 uM *dl*-2-amino-5-phosphonovaleric acid (APV) and 100 μM ketamine were added to the culture medium to protect cells from receptor-mediated excitotoxicity. Cells were used for recordings within 48 hr following transfection. Antagonists were removed before recording by extensive washing.

Electrophysiological recording

Whole-cell patch-clamp recording was performed at room temperature using an Axopatch 1D or 200B amplifier (Axon Instruments Inc., Foster City, CA, USA) as described previously (Ren *et al*., 2012). Gigaohm seals were obtained using patch-pipettes with tip resistances of 2 - 4 MΩ, and series resistances of 1 - 5 MΩ were compensated by 80%. Cells were voltage-clamped at -50 mV and superfused in an external recording solution containing (in mM): 150 NaCl, 5 KCl, 0.2 CaCl2, 10 HEPES, 10 glucose, and 20 sucrose. The ratio of added HEPES free acid and sodium salt was calculated to result

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

in a solution pH of 7.4 (Buffer Calculator, R. Beynon, University of Liverpool; available at www.liv.ac.uk/buffers); final pH was adjusted, if necessary, using HCl or NaOH. Low Ca^{2+} was used to minimize NMDA receptor inactivation, and EDTA, 10 μM, was included to eliminate the fast current relaxation due to high affinity Zn^{2+} inhibition (Low *et al.*, 2000; Zheng *et al.*, 2001). Solutions of agonists and ethanol were prepared fresh daily and applied to cells using a stepper motor-driven solution exchange apparatus (Warner Instruments, Hamden, CT, USA) and three-barrel square glass tubing of internal diameter 600 μm. The intracellular recording solution (patch-pipette) contained (in mM) 140 CsCl, 2 Mg4ATP, 10 BAPTA, and 10 HEPES. The pH was adjusted to 7.4 using HCl or NaOH, and the osmolarity to 310 mOsmol/kg using sucrose. In glutamate concentration-response experiments, cells were lifted off the surface of the dish after obtaining a gigaohm seal to increase the speed of solution exchange. The 10 - 90% rise time for solution exchange under these conditions is ~1.5 ms (Ren *et al*., 2003a). 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).

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, and E_{max} is the maximal current amplitude. Statistical differences among concentration-response curves were determined by comparing log transformed IC_{50} or EC_{50} values from fits to data obtained from individual cells using one-way analysis of variance (ANOVA) followed by the Dunnett test. All values are reported as means ± S.E.M.

Significant interactions among mutants at multiple positions were determined by two-way ANOVA and by mutant cycle analysis. Two-way ANOVA of log-transformed ethanol IC_{50} or glutamate EC_{50} values was performed using the effect of substitution at each of two positions as the two dimensions of the analysis, such that a statistically-significant interaction between these dimensions in the ANOVA would indicate that the amino acid side chain at one position

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

could influence the effect of the side chain at the other position on receptor function. Mutant cycle analysis was performed essentially as described by Venkatachalan and Czajkowski (2008). Tryptophan substitution mutations were introduced singly and in combination at positions in GluN1 and GluN2B subunits proposed to interact, and ethanol IC_{50} and glutamate EC_{50} were determined in each mutant. The interaction free energy, $\Delta\Delta G_{INT}$, for mutations at two positions is the difference in apparent free energy 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). Alternatively, $\Delta\Delta G_{INT}$ may be considered as a comparison between the apparent energy change due to the dual mutant and that due to both of the single mutants, such that nonzero values of $\Delta\Delta G_{INT}$ indicate an interaction between the positions. Interaction free energies among mutated positions were calculated using natural logarithms (ln) of either ethanol IC_{50} or glutamate EC_{50} values obtained from wild-type and mutant subunit combinations, using the equation $\Delta\Delta G_{INT} = RT\ [\ln(WT) + \ln(mut1,mut2) - \ln(mut1) - \ln(mut2)]$. A statistically significant difference between $\Delta\Delta G_{INT}$ and zero energy was taken to indicate an interaction between the two positions. Statistically significant differences were determined by using one sample *t* tests, with degrees of freedom $df = N_{WT} + N_{MUT1} + N_{MUT2} + N_{MUT1,MUT2} - 4$, NX equal to the number of cells used for each combination of wild-type and mutant subunits, and S.E.M. determined from propagated errors.

Materials

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

Results

Single mutations in the M3 and M4 domains of GluN1 and GluN2B subunits alter ethanol inhibition of NMDA receptors.

Previous work in this laboratory has identified significant interactions at four pairs of positions in the M3 and M4 domains of the GluN1 and GluN2A subunits with respect to ethanol inhibition and receptor kinetics (Ren *et al*., 2012). We have also recently shown that in the GluN2B subunit, only two positions corresponding to GluN2A alcohol-sensitive positions, F637 and G826, regulated ethanol sensitivity (Zhao *et al.*, 2015). In the present study, we first tested putative sites of ethanol action in the M3-M4 domains of the GluN1 subunit when expressed with the GluN2B subunit using a tryptophan-scanning approach (Fig 1). All of the tryptophan substitution mutants we tested in this study yielded functional NMDA receptors, although some mutations in the GluN1 and GluN2B subunits noticeably changed receptor characteristics, such as deactivation or macroscopic desensitization (Fig 2A). All tryptophan substitutions in GluN1 M3 showed significantly decreased ethanol sensitivity, while tryptophan substitution at any of the four positions in GluN1 M4 did not alter ethanol sensitivity (Fig 2B, C).

Dual mutations in the M3 and M4 domains of GluN1 and GluN2B subunits alter ethanol inhibition of NMDA receptors.

Next we tested the ethanol sensitivity of receptors with tryptophan substitutions in both the GluN1 and GluN2B subunits at positions in the M3-M4 domain interfaces that are predicted to interact based on our previous study in GluN1/GluN2A NMDA receptors (Ren et al., 2012). We found that all mutant combinations tested formed functional NMDA receptors, with the exception of GluN1(A821W)/GluN2B(F637W) (*not shown*). In some cases the mutations altered receptor function (Fig 3A). In particular, the two

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

combinations involving the GluN1(G638W) mutant affected receptor kinetics, noticeably changing the onset and offset rates of ethanol inhibition, and the deactivation rate. Recordings from cells expressing this subunit also had an erratic appearance that was apparently due to slow fluctuations in current amplitude. Preliminary results suggest that these changes are due at least in part to a prolongation of mean open time (Y. Zhao, *unpublished results)*. Four out of ten dual mutant combinations tested showed significantly altered ethanol sensitivity compared with the wild-type receptor. In some cases, dual mutations in both the GluN1 and GluN2B subunits influenced ethanol sensitivity in a manner that was non-additive compared to the individual mutations, which indicates a functional interaction at these two positions in mediating the action of the ethanol on the receptor (Fig 3B, 3C).

Positions in the M3 and M4 domains of GluN1 and GluN2B subunits can interact to regulate ethanol inhibition of NMDA receptors.

A previous study from this laboratory reported intersubunit interactions between M3 and M4 domain positions in the GluN1 and GluN2A subunits (Ren *et al*., 2012). In order to test for possible interactions among the corresponding positions in the GluN1 and GluN2B subunits, we used both two-way analysis of variance (ANOVA) on log-transformed ethanol IC_{50} values and mutant cycle analysis. We found that each of the two positions in the GluN1 M3 domain interacted with two positions in GluN2B M4. Using both types of analysis, we observed significant interactions with respect to ethanol sensitivity between GluN1(Gly638) and either Met824 or Leu825 in GluN2B, and between GluN1(Phe639) and either Leu825 or Gly826 in GluN2B (Fig. 4 and Table1). We also observed interactions of the GluN2B M3 positions tested with multiple positions in the GluN1 M4 domain. We detected significant interactions of GluN2B(Phe637) with the GluN1 residues Met818 or Val820, but not Leu819 (Fig. 5 and Table1). In contrast, there were no significant interactions detected between GluN2B(Phe638) and any of the three positions tested in the GluN1 subunit M4 domain.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Positions in the M3 and M4 domains of GluN1 and GluN2B subunits can interactively regulate glutamate potency.

Studies from this laboratory have shown that positions in the M3 and M4 domains of different subunits can interactively regulate receptor function (Ren *et al*., 2008; Ren *et al*., 2012). In this study, we tested three pairs of mutants which exhibited significant interactions in regulating ethanol action. Among these combinations, we tested for interactions with respect to glutamate peak and steady-state current EC_{50} values by using two-way analysis of variance as well as mutant cycle analysis. Of the three mutant combinations tested, glutamate peak current EC_{50} was altered in GluN1(G638W)/GluN2B(M824W), and glutamate steady-state current EC_{50} was altered in both GluN1(G638W)/GluN2B(M824W) and GluN1(M818W)/GluN2B(F637W) (Fig. 6). We observed significant interactions among all three pairs tested with respect to glutamate peak current EC_{50} (Fig. 7 and Table 2), and in two pairs with respect to glutamate steady-state current EC_{50} (Fig. 8 and Table 3).

Interaction of GluN1(Gly638) and GluN2B(Met824) in regulation of channel desensitization.

A previous study from this laboratory reported that a tryptophan mutation at position 823 in the M4 domain of the GluN2A subunit can markedly increase desensitization (Ren *et al.*, 2003a). In the present study, tryptophan substitution at GluN2B(Met824) also significantly increased macroscopic desensitization (Fig. 2A), as assessed by using steady-state to peak current ratio (Iss:Ip), whereas tryptophan substitution at GluN1(Gly638) did not alter desensitization (Fig. 9A). However, the effect of the GluN2B(Met824W) mutation on desensitization was partially reversed by coexpression with the GluN1(Gly638W) mutant subunit. Both two-way analysis of variance and mutant cycle analysis of steady-state to peak current ratios indicated a significant interaction between these positions with respect to apparent desensitization (Fig. 9B,C).

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Discussion

We and others have previously shown that substitutions at positions in the M3 and M4 domains of the GluN1 and GluN2B subunits can change NMDA receptor ethanol sensitivity (Ronald *et al.*, 2001; Smothers and Woodward, 2006; Ren *et al.*, 2012; Zhao *et al.*, 2015), and that alcoholsensitive positions in the M domains of the GluN1 and GluN2A subunits can functionally interact (Smothers and Woodward, 2006; Ren *et al.*, 2008, 2012; Xu *et al.*, 2015). In the present study, we have found that mutations in the M3, but not M4, domain of the GluN1 subunit regulate ethanol sensitivity when combined with the GluN2B subunit, and have demonstrated that introduction of dual tryptophan substitutions into positions in the GluN1 and GluN2B subunits at the M3 and M4 intersubunit interfaces can reveal functional interactions among these positions with respect to regulation of ethanol sensitivity and ion channel function.

As in a recent study from this laboratory (Ren *et al.*, 2012), in the present study we have used both two-way analysis of variance and dual mutant cycle analysis to test for interactions between positions at the NMDA receptor M3 and M4 domain intersubunit interfaces. Both tests use log-transformed ethanol IC_{50} values or glutamate EC_{50} values to determine whether the effects of mutations at two positions are independent. We and others have previously used mutant cycle analysis to indicate side-chain interactions regulating agonist or inhibitor potency (Kash *et al.*, 2003; Venkatachalan and Czajkowski, 2008; Laha and Wagner, 2011). In the present study, as in a previous study (Ren *et al.*, 2012), we used tryptophan substitution to detect interactions between positions, rather than alanine substitution, which is typically used (Venkatachalan and Czajkowski, 2008; Laha and Wagner, 2011). Although tryptophan substitution could introduce side-chain interactions that are not normally present, we used it in the present study because our previous work has shown tryptophan to be the substituent most likely to alter ethanol sensitivity and ion channel function. Furthermore, if the cavities bounded by the tested positions form ethanol binding sites in GluN1/GluN2B receptors, as we have proposed for GluN1/GluN2A receptors (Ren *et al.*, 2012), dual tryptophan substitutions would be most likely to exclude the binding of ethanol.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

We recently reported that only two of four positions in the GluN2B subunit corresponding to alcohol-sensitive positions in the GluN2A subunit regulated ethanol sensitivity (Ren *et al.*, 2012; Zhao *et al*., 2015). In contrast, we found in the present study that the positions in the GluN1 subunit regulating alcohol sensitivity are the same whether it is expressed with the GluN2A or GluN2B subunit. Tryptophan substitution at either F638 or F639 in the GluN1 M3 domain, but not at any of four positions from 818-821 in the GluN1 M4 domain, significantly altered ethanol IC_{50} values in GluN1/GluN2B NMDA receptors, which agrees with previous results in GluN1/GluN2A receptors (Smothers and Woodward, 2006; Ren *et al.*, 2012). Similar results were obtained using cysteine substitutions (Xu *et al.*, 2015), although alanine substitution at GluN1(L819) was reported to increase ethanol sensitivity in NMDA receptors containing GluN2A, 2B, or 2C subunits (Smothers and Woodward, 2006). The results of the present study, taken together with those of previous studies, suggest that the role of alcohol-sensitive positions in the GluN1 subunit is not strongly dependent on the coexpressed GluN2 subunit.

Although the influence of GluN1 M3 and M4 domain residues on alcohol sensitivity was similar when expressed with GluN2A or GluN2B subunits, interactions among positions at the M3-M4 domain intersubunit interfaces appear to differ in GluN1/GluN2A and GluN1/GluN2B NMDA receptors. In GluN1/GluN2B subunit-containing receptors, we did not detect significant interactions among all predicted M3-M4 domain positions. We previously reported that GluN1(Leu819) and GluN2A(Phe637) could interactively regulate ethanol sensitivity (Ren *et al*., 2012); however, we did not find an interaction between GluN1(Leu819) and the cognate position GluN2B(Phe638) with respect to ethanol sensitivity. The explanation for this may involve differences in the M3 position in GluN2A and GluN2B. Although GluN2A(Phe637) strongly regulates ethanol action on the NMDA receptor (Ren *et al*., 2007), GluN2B(Phe638) does not (Zhao *et al.*, 2015). These differences agree with our recent findings showing differences in ethanol action on the GluN2A and GluN2B subunits, despite high sequence homology in the M3 and M4 domains (Zhao *et al.*, 2015). We also observed differences among positions in GluN2A and GluN2B regarding multiple interactions with GluN1

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

residues (Fig. 10). Out of four positions in GluN2A that regulate ethanol sensitivity, only Phe637 in M3 interacted with two positions in GluN1 M4, and one interaction appeared to be stronger than the other (Ren *et al.*, 2012), whereas the cognate residue in GluN2B, Phe638, did not interact with any GluN1 position. Both positions in the GluN1 M3 domain interacted equivalently with two positions in the GluN2B M4 domain, the more distal of which, G826, is located approximately 8.6 Å away (measuring from the nearest atom) in the native protein. GluN2B(F637) in M3 significantly interacted with two positions in GluN1 M4, Met818 and Val820. Interestingly, GluN2B(F637) did not interact with its predicted opposing side chain, GluN1(Leu819), but interacted significantly with GluN1(M818), which in the native protein is located on the opposite face of the alpha-helix at a distance of 12.8 Å. The observation that the interaction of GluN2B(F637) with GluN1(M818) appeared to be weaker than that with GluN1(V820) likely reflects the greater distance between these positions. Furthermore, the interactions observed between distant side chains, which in some cases are located on opposite helical faces, most likely involve long-distance functional changes (Ren *et al*., 2008; Ren *et al*., 2012), or to additional positions that interact with one or both members of the pair (Xu *et al.*, 2015). The reason for the presence of these long-distance interactions with respect to ethanol inhibition in GluN2B, but not GluN2A, receptors is not clear, but given the high sequence homology in these regions, may result from subtle structural changes (Zhao *et al.*, 2015), differences in gating (Banke *et al.*, 2003; Erreger *et al.*, 2005), or perhaps differences in ethanol action between the subunit types.

Because ethanol can exert its action at multiple positions in the NMDA receptor, it is likely that those positions would interact functionally to regulate ethanol sensitivity. The first studies demonstrated that residues within the same subunit may interact with each other to modulate ethanol action. Smothers and Woodward (2006) demonstrated that alanine substitution at GluN1(Phe639) significantly reduced ethanol sensitivity, and that tryptophan substitutions at certain positions in the GluN1 subunit M4 domain could reverse the effect of the GluN1(Phe639) alanine mutant. Similarly, a study from this laboratory demonstrated that Phe637 and Met823 in the GluN2A subunit can interactively regulate ethanol sensitivity as

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

well as NMDA receptor function (Ren *et al*., 2008). We recently reported significant interactions with respect to ethanol action between pairs of residues in the M3 domain of one subunit type and the M4 domain of the other in GluN1/GluN2A receptors (Ren *et al*., 2012). Pairs of side chains in that study were tested based on the predicted proximity of their cognate positions in the solved structure of the GluA2 subunit (Sobolevsky *et al*., 2009); the same interactions in the M3 and M4 domains of the GluN1 and GluN2B subunits would be predicted from the recently-published structure of the GluN1/GluN2B receptor (Karakas and Furukawa, 2014). Although not all of the predicted interactions in the present study were confirmed, we nevertheless identified a number of pairs of positions that interactively regulated GluN1/GluN2B NMDA receptor ethanol sensitivity. Interestingly, in some cases a mutation at one position that by itself did not affect ethanol sensitivity could reverse the effect of a second mutation at an interacting position. At type 1 sites (GluN1 M3/GluN2B M4; Fig. 10), tryptophan substitution at either G638 or F639 in the GluN1 subunit M3 domain significantly decreased ethanol sensitivity. Although tryptophan substitution at M824 or L825, the respective nearest neighboring positions in the GluN2B subunit M4 domain, had no effect on ethanol IC_{50} values, these mutations could reverse the effects of GluN1 M3 mutations on ethanol sensitivity: in NMDA receptors bearing dual tryptophan mutations at G638/M824 or F639/L825, ethanol IC_{50} values did not differ from that of the wild-type receptor. Similar results were observed for mutations at the type 2 site, although only the GluN2B(F637) position regulated ethanol sensitivity. These results are similar to those we obtained previously in GluN1/GluN2A subunits, in which intersubunit interactions could reverse the effects of mutations at ethanol-sensitive positions (Ren *et al.*, 2012). The simplest interpretation of our present findings for pairs of adjacent positions at intersubunit interfaces is that mutations at positions that do not by themselves affect ethanol sensitivity can oppose changes in ethanol sensitivity at interacting positions by altering ethanol binding to these sites. For pairs of positions that are not in close proximity, mutations at positions that by themselves do not influence ethanol sensitivity may nevertheless introduce forces on the M domain helices that oppose the action of ethanol.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

A number of studies have reported that ethanol can influence desensitization states of ligandgated ion channels (Moykkynen *et al*., 2003, 2009; Dopico and Lovinger, 2009). In NMDA receptors, the M3 and M4 domains are both important for ion channel gating (Jones *et al*., 2002; Sobolevsky *et al*., 2002; Ren *et al*., 2003a; Schorge and Colquhoun, 2003; Yuan *et al*., 2005; Blank and VanDongen, 2008; Chang and Kuo, 2008), and studies from this and other laboratories have shown that mutations at ethanol-sensitive positions in the M3 and M4 domains of GluN1 and GluN2A subunits can alter ion channel kinetics, including agonist affinity, desensitization, and mean open time (Ronald *et al*., 2001; Ren *et al*., 2003a, 2003b, 2007, 2008, 2013; Smothers and Woodward, 2006). In the present study, we observed that glutamate peak and steady-state current EC_{50} values were altered following tryptophan mutagenesis into individual positions or pairs of positions in the M domains. Because all of the tested positions are at a considerable distance from the ligand-binding domain (Low *et al*., 2003; Yuan *et al.*, 2005; Sobolevsky *et al.*, 2007, 2009), the changes we identified in glutamate EC_{50} among these mutants most probably result from modifications in ion channel gating that reciprocally affect ligand binding. A previous study in this laboratory demonstrated that altered glutamate steady-state EC_{50} values in mutants at GluN2A(Met823) were highly correlated with changes in desensitization, which was most likely due to agonist trapping in one or more long-lived closed states (Ren *et al*., 2003a). However, this is not the case in the present study, because in the majority of mutants glutamate steady-state current EC_{50} values were altered without a corresponding change in desensitization. The precise changes in ion channel gating that underlie the changes in affinity thus remain unclear at present, but may involve changes in dwell times of either open states or short-lived closed states. Whatever the nature of the kinetic changes that accompany M domain mutations, they appear to be interactively regulated. Three pairs of residues that interact to regulate ethanol sensitivity also interacted to regulate glutamate peak current EC_{50}, and two pairs of residues interactively regulated glutamate steady-state current EC_{50}. We also observed an interaction with respect to macroscopic desensitization for one pair of residues, GluN1(Gly638) and GluN2B(Met824). Tryptophan substitution at GluN2B(Met824) markedly increased desensitization, as was observed at the cognate position in the GluN2A subunit (Ren *et al.*, 2003a).

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Tryptophan substitution at GluN1(Gly638) had no effect on desensitization when expressed with wild-type GluN2B subunits, but partially reversed the effect of the GluN2B(Met824Trp) mutant. Mutant cycle analysis of maximal steady-state to peak current ratio revealed a significant interaction between these positions. These results suggest that the side chains of these two residues are able to interact, at least when tryptophan is introduced into both positions, in a manner that influences ion channel gating.

In summary, the results of this study identified multiple interactions with respect to ethanol inhibition and ion channel gating among positions at the intersubunit interfaces of the M3 and M4 domains forming putative sites of ethanol action in the GluN1/GluN2B NMDA receptor. Despite both a high degree of sequence homology in the M domains and similar ethanol sensitivity, these interactions differ in the GluN2A and GluN2B subunits.

Author Contributions

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

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References

Banke, TG & Traynelis, SF. (2003). Activation of NR1/NR2B NMDA receptors. *Nat Neurosci*, **6**, 144-152.

Blanke, ML & VanDongen, AMJ. (2008). The NR1 M3 Domain Mediates Allosteric Coupling in the N-Methyl-D-aspartate Receptor. *Mol Pharmacol*, **74**, 454-465.

Boyce-Rustay, JM & Holmes, A. (2005). Functional roles of NMDA receptor NR2A and NR2B subunits in the acute intoxicating effects of ethanol in mice. *Synapse*, **56**, 222-225.

Chandrasekar, R. (2013). Alcohol and NMDA receptor: current research and future direction. *Front Mol Neurosci*, **6**, 14.

Chang, HR & Kuo, CC. (2008). The Activation Gate and Gating Mechanism of the NMDA Receptor. *J Neurosci*, **28**, 1546-1556.

Chazot, PL. (2004). The NMDA receptor NR2B subunit: a valid therapeutic target for multiple CNS pathologies. *Curr Med Chem*, **11**, 389-396.

Dopico, AM & Lovinger, DM. (2009). Acute alcohol action and desensitization of ligand-gated ion channels. *Pharmacol Rev*, **61**, 98-114.

Erreger, K & Traynelis, SF. (2005). Allosteric interaction between zinc and glutamate binding domains on NR2A causes desensitization of NMDA receptors. *J Physiol*, **569**, 381-393.

Gass, JT & Olive, MF. (2008). Glutamatergic substrates of drug addiction and alcoholism. *Biochem Pharmacol*, **75**, 218-265.

Gogas, KR. (2006). Glutamate-based therapeutic approaches: NR2B receptor antagonists. *Curr Opin Pharmacol*, **6**, 68-74.

Holmes, A, Spanagel, R & Krystal, JH. (2013). Glutamatergic targets for new alcohol medications. *Psychopharmacol*, **229**, 539-554.

Honse, Y, Ren, H, Lipsky, RH & Peoples, RW. (2004). Sites in the fourth membrane-associated domain regulate alcohol sensitivity of the NMDA receptor. *Neuropharmacol*, **46**, 647-654.

Jones, KS, VanDongen, HMA & VanDongen, AMJ. (2002). The NMDA receptor M3 segment is a conserved transduction element coupling ligand binding to channel opening. *J Neurosci*, **22**, 2044-2053.

Karakas, E & Furukawa, H. (2014). Crystal structure of a heterotetrameric NMDA receptor ion channel. *Science*, **344**, 992-997.

Kash, TL, Jenkins, A, Kelley, JC, Trudell, JR, & Harrison, NL. (2003). Coupling of agonist binding to channel gating in the GABAA receptor. *Nature* **421**, 272-275.

Kash, TL, Baucum, AJ, Conrad, KL, Colbran, RJ & Winder, DG. (2009). Alcohol exposure alters NMDAR function in the bed nucleus of the stria terminalis. *Neuropsychopharmacology*, **34**, 2420-2429.

Kash, TL, Matthews, RT & Winder, DG. (2008). Alcohol inhibits NR2B-containing NMDA receptors in the ventral bed nucleus of the stria terminalis. *Neuropsychopharmacology*, **33**, 1379-1390.

Koob, GF. (2003). Alcoholism: allostasis and beyond. *Alcohol Clin Exp Res*, **27**, 232-243.

Krystal, JH, Petrakis, IL, Webb, E, Cooney, NL, Karper, LP, Namanworth, S, Stetson, P, Trevisan, LA & Charney, DS. (1998). Dose-related ethanol-like effects of the NMDA antagonist, ketamine, in recently detoxified alcoholics. *Arch Gen Psychiatry*, **55**, 354-360.

Krystal, JH, Petrakis, IL, Mason, G, Trevisan, L & D'Souza, DC. (2003). N-methyl-D-aspartate glutamate receptors and alcoholism: reward, dependence, treatment, and vulnerability. *Pharmacology & Therapeutics*, **99**, 79-94.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Laha, KT & Wagner, DA. (2011). A state-dependent salt-bridge interaction exists across the □/□ intersubunit interface of the GABAA receptor. *Mol Pharmacol* **79**, 662-671.

Lee, CH, Lu, W, Michel, JC, Goehring, A, Du, J, Song, X & Gouaux, E. (2014). NMDA receptor structures reveal subunit arrangement and pore architecture. *Nature*, **511**, 191-197.

Lovinger, DM, White, G & Weight, FF. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science*, **243**, 1721-1724.

Low, CM, Lyuboslavsky, P, French, A, Le, P, Wyatte, K, Thiel, WH, Marchan, EM, Igarashi, K, Kashiwagi, K, Gernert, K, Williams, K, Traynelis, SF & Zheng, F. (2003). Molecular determinants of proton-sensitive N-methyl-D-aspartate receptor gating. *Mol Pharmacol*, **63**, 1212-1222.

Low, CM, Zheng, F, Lyuboslavsky, P & Traynelis, SF. (2000). Molecular determinants of coordinated proton and zinc inhibition of *N*-methyl-D-aspartate NR1/NR2A receptors. *Proc Natl Acad Sci USA*, **97**, 11062-11067.

Moykkynen, T, Korpi, ER & Lovinger, DM. (2003). Ethanol inhibits alpha-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function in central nervous system neurons by stabilizing desensitization. *J Pharmacol Exp Ther*, **306**, 546-555.

Moykkynen, TP, Coleman, SK, Keinanen, K, Lovinger, DM & Korpi, ER. (2009). Ethanol increases desensitization of recombinant GluR-D AMPA receptor and TARP combinations. *Alcohol*, **43**, 277-284.

Nagy, J. (2004). The NR2B subtype of NMDA receptor: a potential target for the treatment of alcohol dependence. *Curr Drug Targets CNS Neurol Disord*, **3**, 169-179.

Peoples, RW. (2003). Alcohol Actions on Glutamate Receptors.In *Glutamate and Addiction.* ed. Herman,B.H., Frankenheim,J., Litten,R.Z., Sheridan,P.H., Weight,F.F. & Zukin,S.R. pp. 343-356. Humana Press: Totowa, NJ.

Ren, H, Honse, Y, Karp, BJ, Lipsky, RH & Peoples, RW. (2003a). 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.

Ren, H, Honse, Y & Peoples, RW. (2003b). A site of alcohol action in the fourth membraneassociated domain of the NMDA receptor. *J Biol Chem*, **278**, 48815-48820.

Ren, H, Salous, AK, Paul, JM, Lamb, KA, Dwyer, DS & Peoples, RW. (2008). Functional interactions of alcohol-sensitive sites in the N-methyl-D-aspartate receptor M3 and M4 domains. *J Biol Chem*, **283**, 8250-8257.

Ren, H, Salous, AK, Paul, JM, Lipsky, RH & Peoples, RW. (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.

Ren, H, Zhao, Y, Dwyer, DS & Peoples, RW. (2012). 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. *J Biol Chem*, **287**, 27302-27312.

Ren, H, Zhao, Y, Wu, M & Peoples, RW. (2013). A novel alcohol-sensitive position in the Nmethyl-D-aspartate receptor GluN2A subunit M3 domain regulates agonist affinity and ion channel gating. *Mol Pharmacol*, **84**, 501-510.

Ronald, KM, Mirshahi, T & Woodward, JJ. (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.

Ryan, TJ, Kopanitsa, MV, Indersmitten, T, Nithianantharajah, J, Afinowi, NO, Pettit, C, Stanford, LE, Sprengel, R, Saksida, LM, Bussey, TJ, O'Dell, TJ, Grant, SG & Komiyama, NH. (2013). Evolution of GluN2A/B cytoplasmic domains diversified vertebrate synaptic plasticity and behavior. *Nat Neurosci*, **16**, 25-32.

Schorge, S & Colquhoun, D. (2003). Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. *J Neurosci*, **23**, 1151-1158.

Smothers, CT & Woodward, JJ. (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.

Sobolevsky, AI, Beck, C & Wollmuth, LP. (2002). Molecular rearrangements of the extracellular vestibule in NMDAR channels during gating. *Neuron*, **33**, 75-85.

Sobolevsky, AI, Rosconi, MP & Gouaux, E. (2009). X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor. *Nature*, **462**, 745-756.

Sobolevsky, AI, Prodromou, ML, Yelshansky, MV & Wollmuth, LP. (2007). Subunit-specific Contribution of Pore-forming Domains to NMDA Receptor Channel Structure and Gating. *The Journal of General Physiology*, **129**, 509-525.

Venkatachalan, SP & Czajkowski, C. (2008). A conserved salt bridge critical for GABAA receptor function and loop C dynamics. *Proc Natl Acad Sci U S A*, **105**, 13604-13609.

Wang, J, Carnicella, S, Phamluong, K, Jeanblanc, J, Ronesi, JA, Chaudhri, N, Janak, PH, Lovinger, DM & Ron, D. (2007). Ethanol Induces Long-Term Facilitation of NR2B-NMDA Receptor Activity in the Dorsal Striatum: Implications for Alcohol Drinking Behavior. *J Neurosci*, **27**, 3593-3602.

Wills, TA, Klug, JR, Silberman, Y, Baucum, AJ, Weitlauf, C, Colbran, RJ, Delpire, E & Winder, DG. (2012). GluN2B subunit deletion reveals key role in acute and chronic ethanol sensitivity of glutamate synapses in bed nucleus of the stria terminalis. *Proc Natl Acad Sci U S A*, **109**, E278-E287.

Woodward, JJ. (1999). Ionotropic glutamate receptors as sites of action for ethanol in the brain. *Neurochem Int*, **35**, 107-113.

Wright, JM, Peoples, RW & Weight, FF. (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.

Xu, M, Smothers, CT & Woodward, JJ. (2015). Cysteine substitution of transmembrane domain amino acids alters the ethanol inhibition of GluN1/GluN2A N-methyl-D-aspartate receptors. *J Pharmacol Exp Ther*, **353**, 91-101.

Yuan, H, Erreger, K, Dravid, SM & Traynelis, SF. (2005). Conserved structural and functional control of N-methyl-D-aspartate receptor gating by transmembrane domain M3. *J Biol Chem*, **280**, 29708-29716.

Zhao, Y, Ren, H, Dwyer, DS & Peoples, RW. (2015). Different sites of alcohol action in the NMDA receptor GluN2A and GluN2B subunits. *Neuropharmacol*.

Zheng, F, Erreger, K, Low, CM, Banke, T, Lee, CJ, Conn, PJ & Traynelis, SF. (2001). Allosteric interaction between the amino terminal domain and the ligand binding domain of NR2A. *Nat Neurosci*, **4**, 894-901.

Table 1. Mutant Cycle Analysis of Ethanol EC_{50}

Mutant Pair (GluN1/GluN2B)	ΔG_1 WT→N1	ΔG_2 WT→N2B	ΔG_3 WT→N1/N2B	ΔG_4 N1→N1/N2B	ΔG_5 N2B→N1/N2B	$\Delta\Delta G_{INT}$	df	Significance
G638W/M824W	0.303	0.0987	-0.0162	-0.319	-0.115	-0.417	20	$P < 0.0001$
G638W/L825W	0.303	-0.0511	0.558	0.255	0.609	0.307	19	$P = 0.0001$
F639W/L825W	0.222	-0.0511	0.0108	-0.212	0.0620	-0.160	21	$P < 0.01$
F639W/G826W	0.222	0.422	0.775	0.552	0.353	0.130	21	$P < 0.05$
M818W/F637W	0.0722	0.543	0.122	0.0503	-0.420	-0.493	21	$P < 0.0001$
L819W/F637W	-0.0605	0.543	0.551	0.611	0.00792	0.0684	20	$P > 0.05$
V820W/F637W	-0.0275	0.543	0.253	0.280	-0.290	-0.262	20	$P < 0.005$
L819W/F638W	-0.0605	0.0112	-0.0352	0.0253	-0.0471	0.0134	16	$P > 0.05$
V820W/F638W	-0.0275	0.0119	0.0667	0.0941	0.0548	0.0823	14	$P > 0.05$
A821W/F638W	-0.177	0.0119	-0.0829	0.0942	-0.0947	0.0823	14	$P > 0.05$

Values of ΔGX in kcal mol-1 are RT [ln(R1 IC_{50} – ln(R2 IC_{50})], where R1 and R2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild-type; N1, GluN1 mutant/GluN2B wild-type; N2, GluN1 widetype/GluN2B mutant; N1/N2B, GluN1 mutant/GluN2B mutant). Values of apparent free energy ΔΔGINT in kcal mol-1 are means ± S.E.M. Values of ΔΔGINT, degrees of freedom (df), and statistical significance of ΔΔGINT were determined as described in the *Methods*.

Table 2. Mutant Cycle Analysis of Glutamate Peak Current (Ip) EC_{50} Mutant Pair (GluN1/GluN2B)

Mutant Pair (GluN1/GluN2B)	ΔG_1 WT→N1	ΔG_2 WT→N2B	ΔG_3 WT→N1/N2B	ΔG_4 N1→N1/N2B	ΔG_5 N2B→N1/N2B	$\Delta\Delta G_{INT}$	df	Significance
G638W/M824W	-1.18	0.0540	-0.326	0.853	-0.380	0.799	20	$P < 0.0001$
F639W/L825W	-0.486	0.170	-0.0457	0.440	-0.216	0.270	20	$P < 0.05$
M818W/F637W	0.276	-0.769	0.0468	-0.230	0.816	0.540	21	$P < 0.001$
L819W/F638W	0.000881	0.131	0.241	0.240	0.110	0.109	17	$P > 0.05$

Values of ΔGX in kcal mol-1 are RT [ln(R1 EC_{50} – ln(R2 EC_{50})], where R1 and R2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild-type; N1, GluN1 mutant/GluN2B wild-type; N2, GluN1 wild-type/GluN2B mutant; N1/N2B, GluN1 mutant/GluN2B mutant). Values of apparent free energy ΔΔGINT in kcal mol-1 are means ± S.E.M. Values of ΔΔGINT, degrees of freedom (df), and statistical significance of ΔΔGINT were determined as described in the *Methods*.

Table 3. Mutant Cycle Analysis of Glutamate Steady-State Current (Iss) EC_{50}

Mutant Pair (GluN1/GluN2B)	ΔG_1 WT→N1	ΔG_2 WT→N2B	ΔG_3 WT→N1/N2B	ΔG_4 N1→N1/N2B	ΔG_5 N2B→N1/N2B	$\Delta\Delta G_{INT}$	df	Significance
G638W/M824W	-1.06	-0.641	-0.984	0.0765	-0.343	0.717	21	$P < 0.05$
F639W/L825W	-0.380	0.112	-0.0998	0.280	-0.212	0.168	20	$P > 0.05$
M818W/F637W	0.397	-0.618	0.183	-0.214	0.801	0.404	21	$P < 0.05$
L819W/F638W	-0.0842	0.0426	-0.0293	0.0549	-0.0719	0.0123	17	$P > 0.05$

Values of ΔGX in kcal mol-1 are RT [ln(R1 EC_{50} – ln(R2 EC_{50})], where R1 and R2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild-type; N1, GluN1 mutant/GluN2B wild-type; N2, GluN1 wild-type/GluN2B mutant; N1/N2B, GluN1 mutant/GluN2B mutant). Values of apparent free energy ΔΔGINT in kcal mol-1 are means ± S.E.M. Values of ΔΔGINT, degrees of freedom (df), and statistical significance of ΔΔGINT were determined as described in the *Methods*.

Figure 1

M3
GluN1 630 RILGMVWA**GF**AMIIVASYTANLAAF GluN2A 631
VSVWA**FF**AVIFLASYTANLAAFMIQ GluN2B 632
VSVWA**FF**AVIFLASYTANLAAFMIQ

M4
GluN1 811 ENMAGVFM**LVA**GGIVAGIF GluN2A 822
Y**MLA**AAMALSLITFIW GluN2B 823
Y**MLG**AAMALSLITFIC

Fig. 1. Positions in the GluN1 and GluN2B subunit M3 and M4 domains constituting putative sites of ethanol action. Partial sequences of the M3 and M4 domains in GluN1 and GluN2B subunits are shown, with positions in GluN2B corresponding to ethanol-sensitive or interacting positions in GluN2A indicated in bold. The location of GluN2B(Phe637), the main position regulating ethanol sensitivity in the GluN2B subunit (Zhao *et al.*, 2015), is indicated by the arrow.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 2

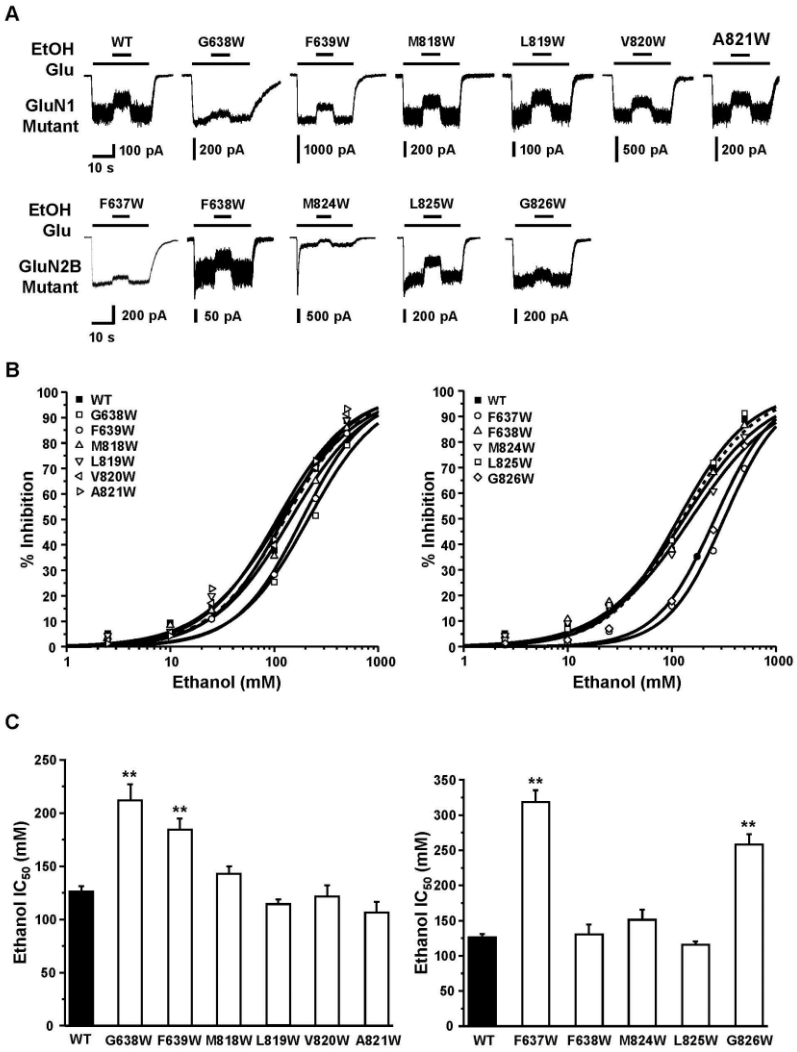

Fig. 2. **Ethanol sensitivity of tryptophan mutant subunits in the M3 and M4 domains of the GluN1 and GluN2B subunits.** *A.* Traces are currents activated by 10 μM glutamate and 50 μM glycine and their inhibition by 100 mM ethanol in cells expressing various single tryptophan substitution mutations in the GluN1 (*upper*) and GluN2B (*lower*) subunits. One-letter amino acid codes are used. *B.* Concentration-response curves show ethanol inhibition of current activated by 10 μM glutamate in the presence of 50 μM glycine in cells expressing various single mutant GluN1 (*left*) and GluN2B (*right*) subunits. Curves shown are the best fits to the equation given in the *Methods*. Data points are means ± S.E. of 5-7 cells. *C.* Bar graphs show average IC_{50} values for ethanol in cells expressing GluN1 (*left*) and GluN2B (*right*) subunits containing individual mutations in the M3 and M4 domains. Asterisks indicate IC_{50} values that differed significantly from the IC_{50} value for wild type GluN1/GluN2B subunits (**$P < 0.01$; ANOVA and Dunnett's test). The black bars show the average ethanol IC_{50} value for the wild-type receptor. Results are means ± S.E. of 5-7 cells.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 3

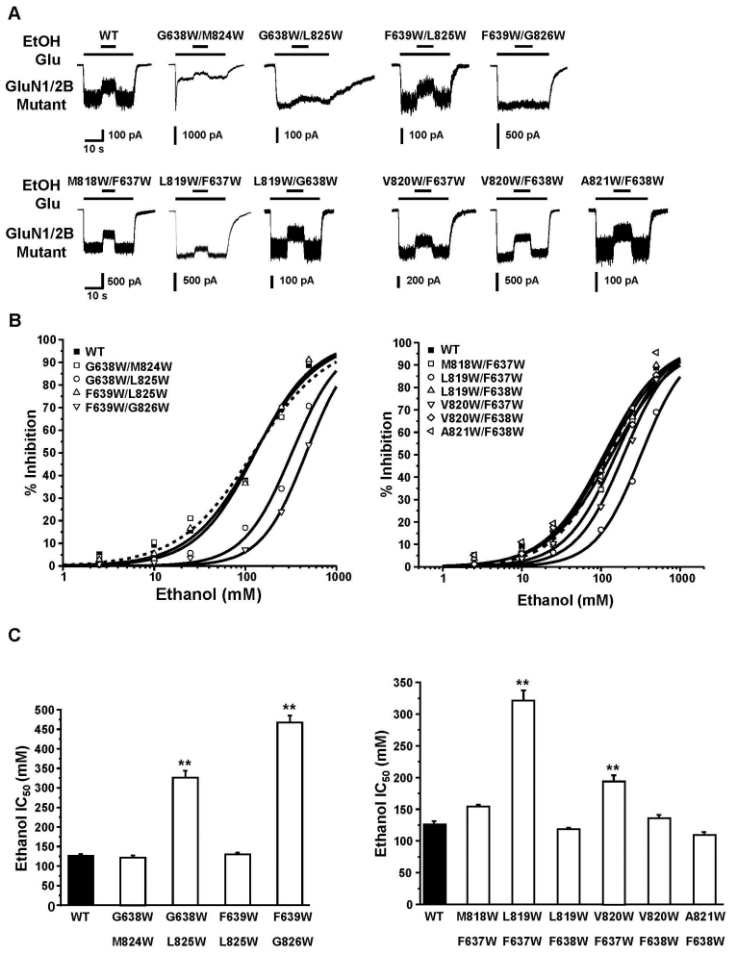

Fig. 3. Ethanol sensitivity of dual tryptophan substitution mutations in the M3 and M4 domains of the GluN1 and GluN2B subunits. *A*. Records are currents activated by 10 μM glutamate and 50 μM glycine and their inhibition by 100 mM ethanol in cells expressing dual mutations in the GluN1 M3 / GluN2B M4 domains (*upper*) and GluN1 M4 / GluN2B M3 domains (*lower*), as indicated. One-letter amino acid codes are used. *B.* Ethanol concentration-response curves for inhibition of glutamate-activated currents in wild-type and mutant receptors. Dual-site substitution mutations in the GluN1 M3 / GluN2B M4 domains are shown on the left, and those in the GluN1 M4 / GluN2B M3 domains are on the right. Data are means ± S.E. of 4-7 cells. Curves shown are the best fits to the equation given in the *Methods*. *C*. Graphs plot average IC_{50} values for ethanol in dual mutations in the GluN1 M3 / GluN2B M4 domains (*left*) and the GluN1 M4 / GluN2B M3 domains (*right*). *Asterisks* indicate IC_{50} values that differed significantly from the IC_{50} value for wild type GluN1/GluN2B subunits (**$P < 0.01$; ANOVA and Dunnett's test). The black bars show the average ethanol IC_{50} value for the wild-type receptor. Results are means ± S.E. of 5-7 cells.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 4

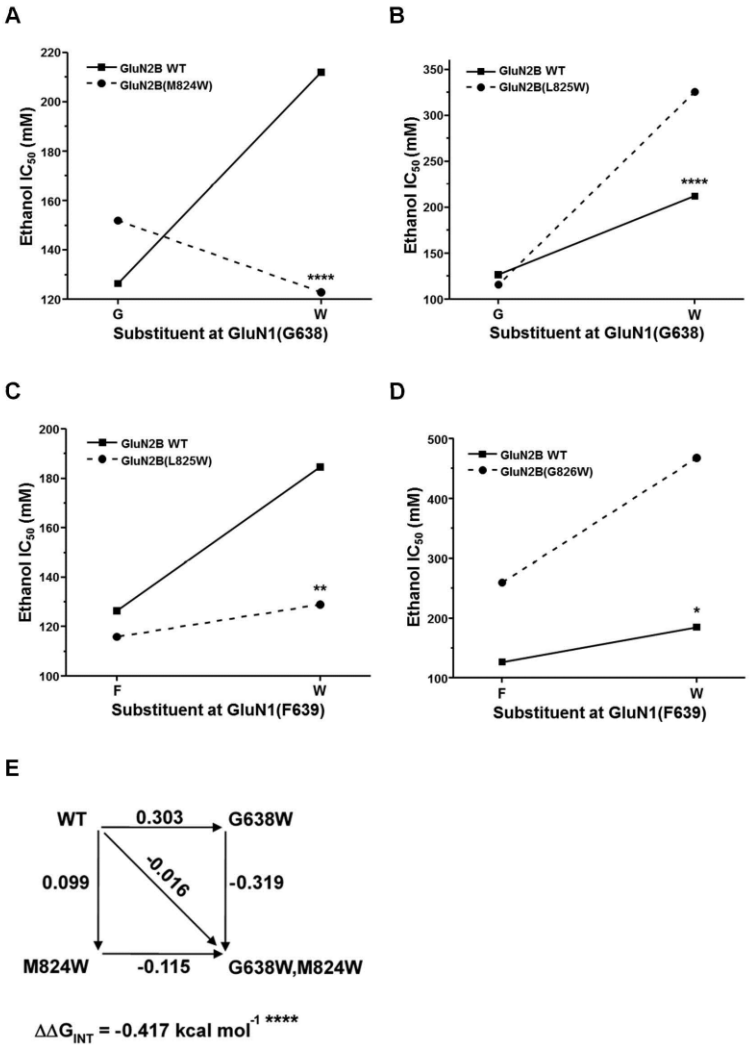

Fig. 4. Positions in the M3 domain of the GluN1 subunit and positions in the M4 domain of the GluN2B subunit interactively regulate NMDA receptor ethanol sensitivity. *A-D*. Graphs plot ethanol IC_{50} values *vs*. the substituent at GluN1(G638) or GluN1(F639) for mutants at GluN2B positions 824-826, as indicated. Significant interactions between positions detected using log-transformed IC_{50} values are indicated by asterisks (*P < 0.05, **P < 0.01, ****P < 0.0001; two-way ANOVA). One-letter amino acid codes are used. *E*. Mutant cycle analysis of ethanol IC_{50} values for the combination GluN1(Gly638)/GluN2B(Met824), which showed a significant interaction with respect to ethanol sensitivity. Apparent free energy values associated with various mutations (Δ*Gx*) are given in kcal mol-1. Asterisks indicate a statistically significant difference of the apparent interaction energy ΔΔ*GINT* from zero energy determined using a one sample *t* test (****P < 0.0001).

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 5

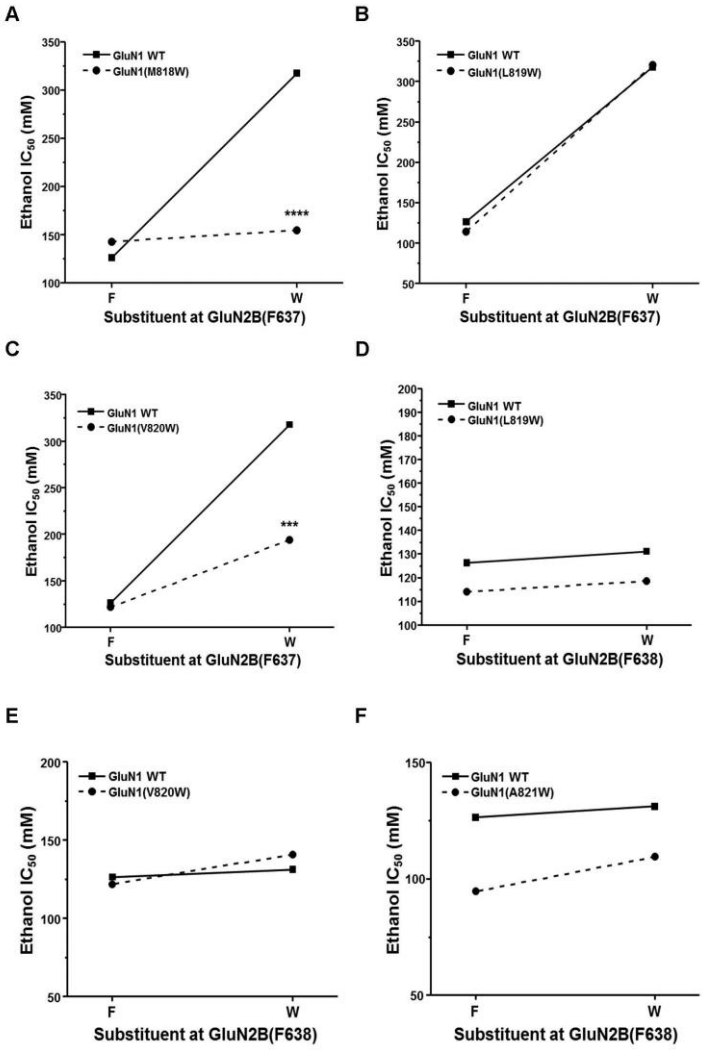

Fig. 5. Positions in the M3 domain of the GluN2B subunit and positions in the M4 domain of the GluN1 subunit interact to regulate NMDA receptor ethanol sensitivity. Graphs plot ethanol IC_{50} values *vs*. the substituent at GluN2B(Phe637) (*A-C*) or GluN2B(Phe638) (*D-F*) for mutants at GluN1 positions 818, 819, 820 and 821, as indicated. Significant interactions detected using log-transformed IC_{50} values are indicated by asterisks (***$P < 0.001$, ****$P < 0.0001$; twoway ANOVA).

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 6

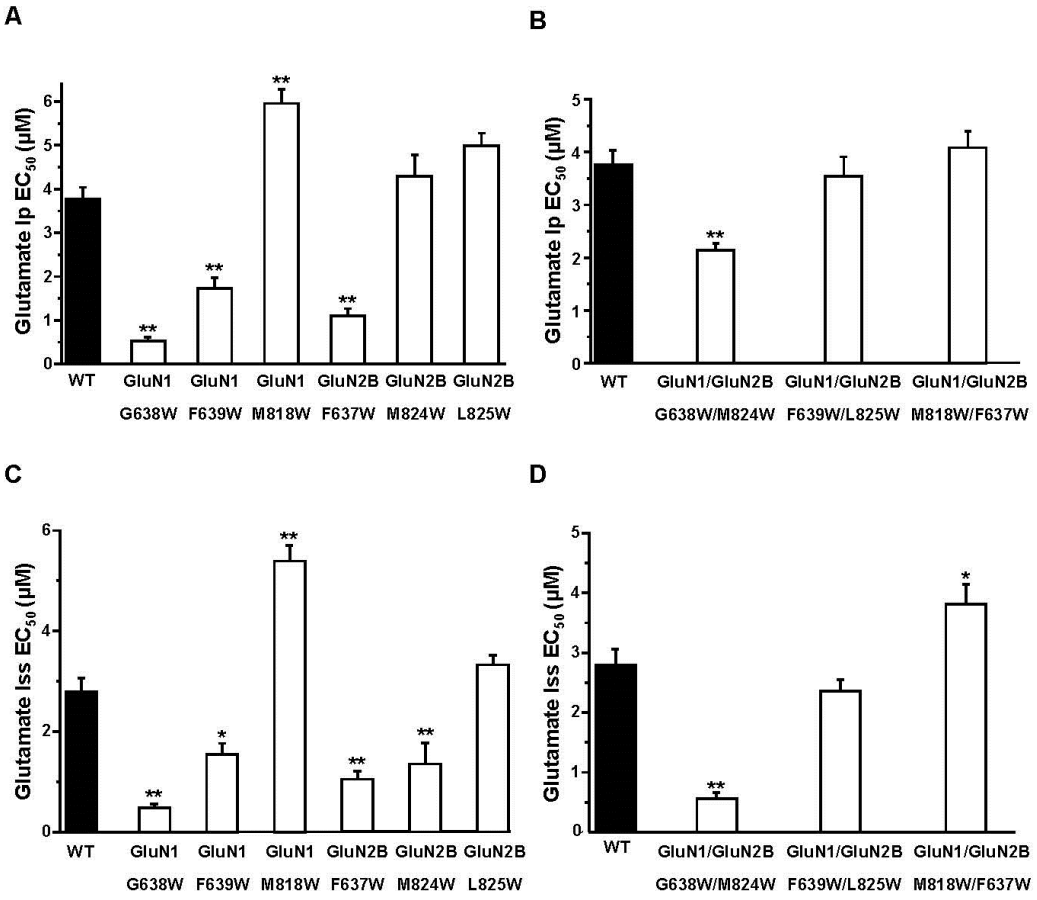

Fig. 6. Tryptophan substitutions in the M3 and M4 domains of the GluN1 and GluN2B subunits alter glutamate potency. Bar graphs plot glutamate EC_{50} values for peak (Ip; *A-B*) and steady-state (Iss; *C-D*) current in lifted cells expressing GluN1 and GluN2B subunits with various substitutions in the M3 and M4 domains. Asterisks indicate EC_{50} values that differ significantly from that of the wild-type GluN1/GluN2B subunit (*$P < 0.05$, **$P < 0.01$; ANOVA and Dunnett's test). Results are the means ± S.E of 5-8 cells. The black bars show the EC_{50} values for the wildtype receptor.

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 7

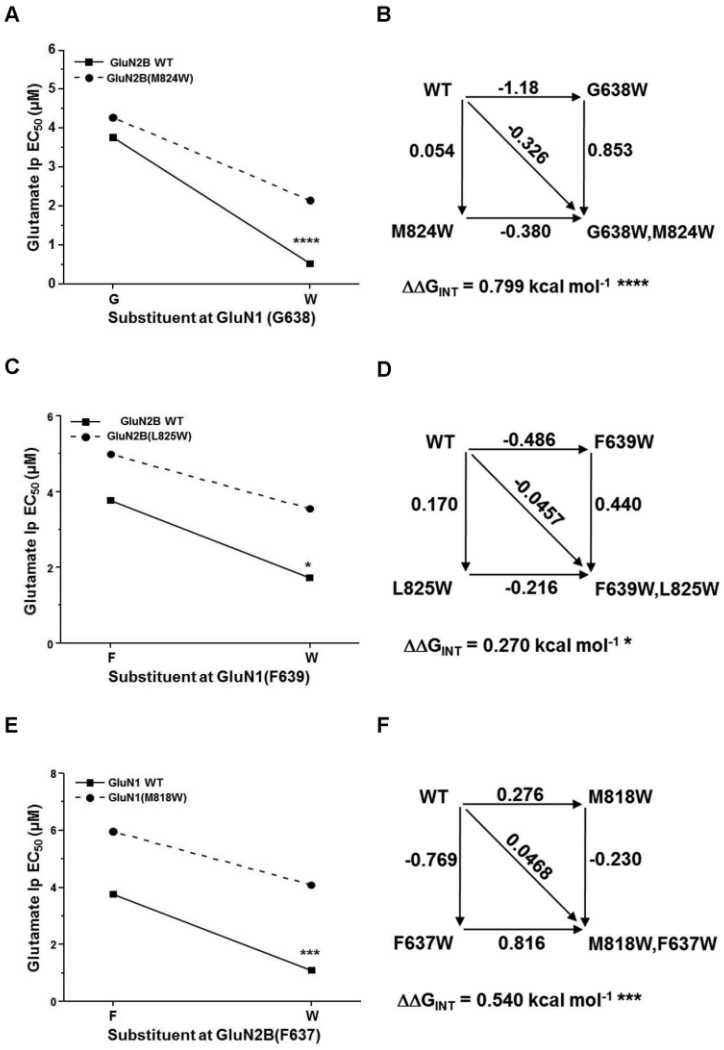

Fig. 7. Positions in the M3 and M4 domains of the GluN1 and GluN2B subunits interactively regulate glutamate peak current EC_{50}. *A,C,E*. Graphs plot glutamate peak current EC_{50} values *vs*. the substituent at position 638, 639 and 818 of GluN1 for mutants at GluN2B positions 824, 825 and 637, as indicated. Asterisks indicate significant interactions detected using logtransformed peak current EC_{50} values (*P < 0.05, ***P < 0.001, ****P < 0.0001; two-way ANOVA). *B,D,F.* Mutant cycle analysis of glutamate peak current EC_{50} values for GluN1/GluN2B mutant combinations. Apparent free energy values associated with various mutations (Δ*Gx*) are given in kcal mol-1. Asterisks indicate a statistically significant difference of the apparent interaction energy ΔΔ*GINT* from zero energy determined using a one sample *t* test (*P < 0.05; ***P < 0.001; ****P < 0.0001).

Figure 8

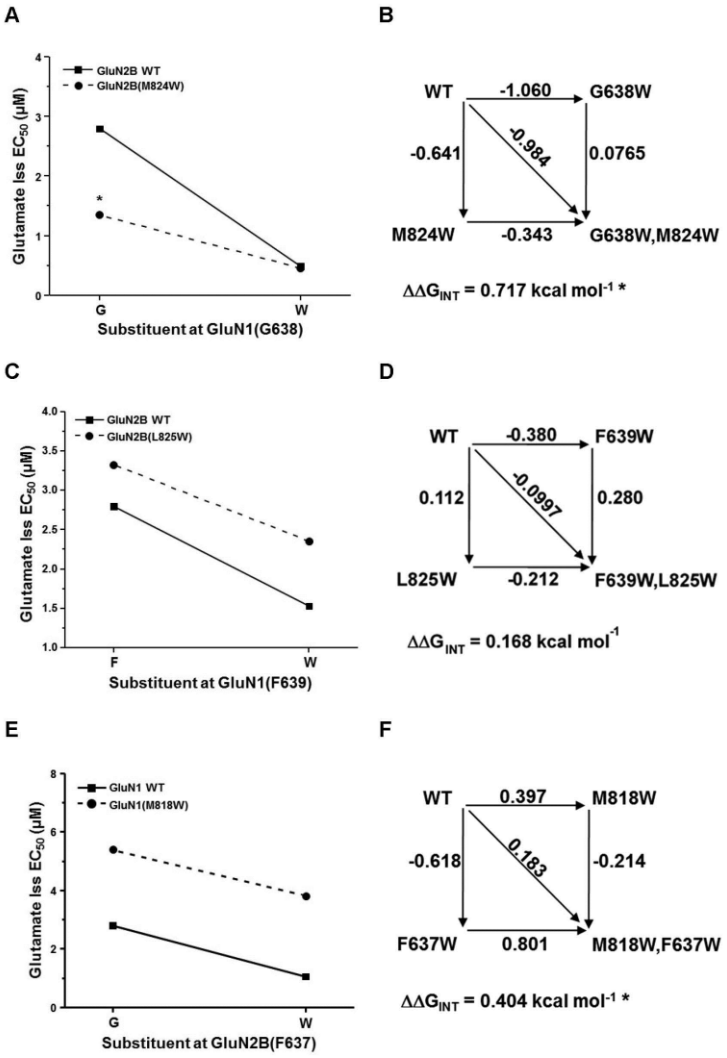

Fig. 8. Positions in the M3 and M4 domains of GluN1 and GluN2B subunits interactively regulate glutamate steady-state current EC_{50}. *A,C,E*. Graphs plot glutamate steady-state current EC_{50} values *vs*. the substituent at position 638, 639 and 818 of GluN1 for mutants at GluN2B positions 824, 825 and 637, as indicated. Asterisks indicate significant interactions detected using log-transformed steady-state current EC_{50} values (*P < 0.05; two-way ANOVA). *B,D,F.* Mutant cycle analysis of glutamate steady-state current EC_{50} values for GluN1/GluN2B mutant combinations. Apparent free energy values associated with various mutations (ΔGx) are given in kcal mol-1. Asterisks indicate a statistically significant difference of the apparent interaction energy $\Delta\Delta GINT$ from zero energy determined using a one sample *t* test (*P < 0.05).

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 9

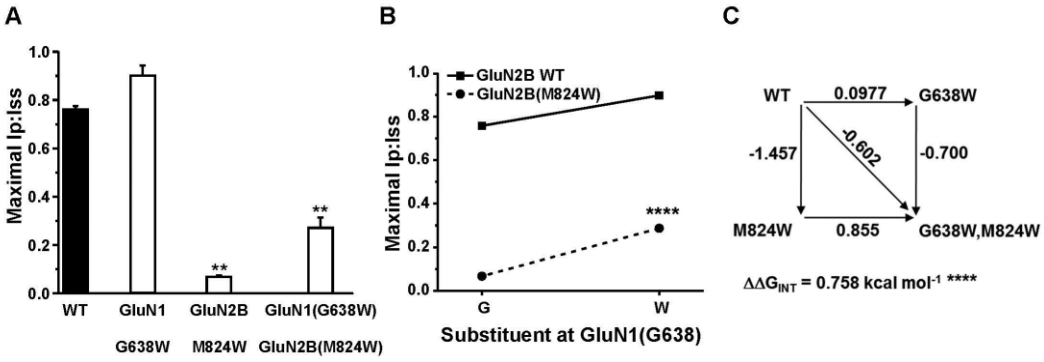

Fig. 9. Positions GluN1(Gly638) and GluN2B(Met824) interact to regulate NMDA receptor macroscopic desensitization. *A.* Bar graph shows maximal steady-state to peak current ratios (Iss : Ip) for current activated by 300 μM glutamate and 50 μM glycine recorded from cells expressing wild-type GluN1/GluN2B, GluN1(Gly638Trp)/GluN2B, GluN1/GluN2B(Met824Trp), and GluN1(Gly638Trp)/GluN2B(Met824Trp) subunits. Statistically significant differences in maximal apparent desensitization from the value for the wild-type receptor are indicated by asterisks (**P < 0.01; ANOVA and Dunnett's test). *B*. Graph plots the maximal steady-state to peak current ratio *vs*. the substituent at GluN1(Gly638) for GluN2B(Met824), as indicated. Asterisks indicate significant interactions detected using values for maximal steady-state to peak current ratio (****P < 0.0001; two-way ANOVA). *C*. Mutant cycle analysis of maximal steadystate to peak current ratios for the positions GluN1(Gly638) and GluN2B(Met824). Apparent free energy values associated with various mutations (Δ*Gx*) are given in kcal mol-1. Asterisks indicate a statistically significant difference of the apparent interaction energy ΔΔ*GINT* from zero energy determined using a one sample *t* test (****P < 0.0001).

British Journal of Pharmacology, Vol. 173, No. 12 (June 2016): pg. 1950-1965. DOI.

Figure 10

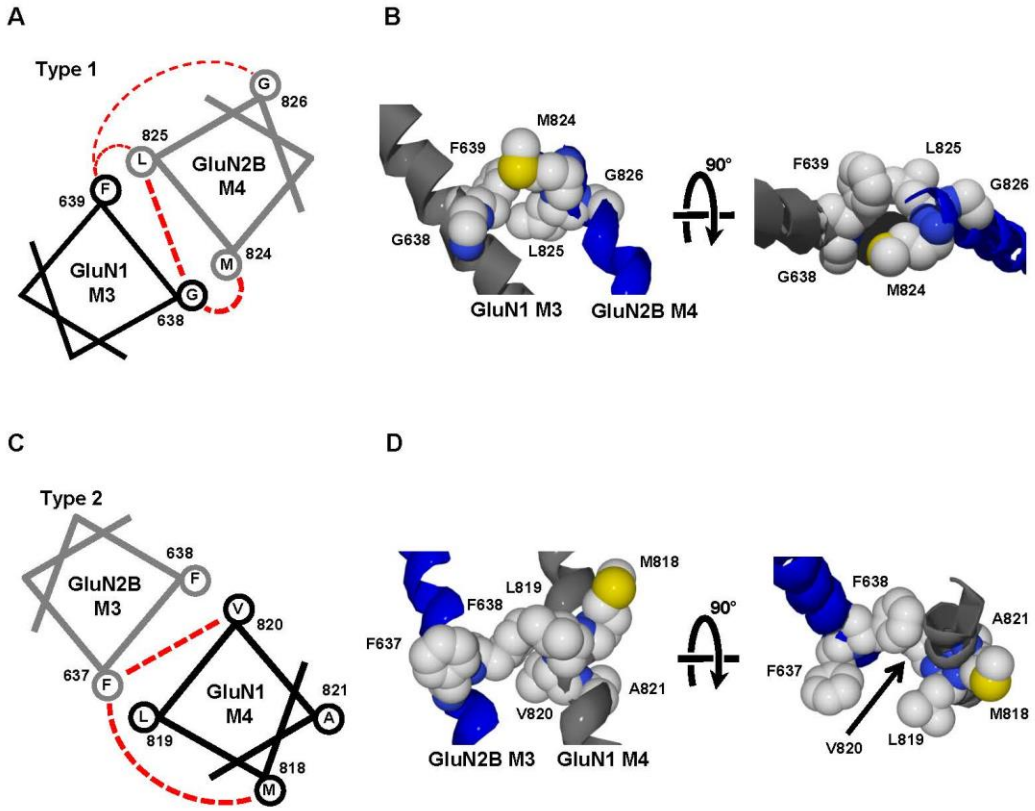

Fig. 10. Positions in the GluN1 and GluN2B subunit M3 and M4 domain intersubunit interfaces that interact to regulate ethanol action. *A,C*, Helical wheel plots of the regions of the GluN1 M3 / GluN2B M4 (type 1) and GluN2B M3 / GluN1 M4 (type 2) interfaces constituting putative sites of ethanol action. Circles represent amino acid positions oriented as in Karakas and Furukawa (2014). One-letter amino acid codes are used. Significant interactions between positions with respect to ethanol sensitivity are indicated by dashed lines; the line thickness represents the apparent relative strength of the interaction as indicated by the level of significance determined by two-way ANOVA and mutant cycle analysis (thin lines, $P <$ 0.01 – 0.05; thick lines, $P <$ 0.0001 - 0.001). *B*, Molecular model of the ethanol site formed by the GluN1 subunit M3 domain (*gray*) and GluN2B subunit M4 domain (*blue*). *D,* Molecular model of the ethanol site formed by the GluN2B subunit M3 domain (*blue*) and GluN1 subunit M4 domain (*gray*).

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