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# Probing NMDA receptor desensitization with the substituted-cysteine accessibility method

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**Abstract**

Several forms of macroscopic NMDA receptor desensitization affect the amplitude and duration of postsynaptic responses. In addition to its functional significance, desensitization provides one means to examine the conformational coupling of ligand binding to channel gating. Segments flanking the ligand-binding domain in the extracellular N-terminus of the NMDA receptor NR2 subunit influence the glycine-independent form of desensitization. The NR2A pre-M1 region, the linker between the glutamate binding domain and the channel pore, plays a critical role in desensitization. Thus we used the substituted-cysteine accessibility method to scan the accessibility of residues in the pre-M1 region and the first transmembrane domain (M1) of NR2A. Cysteine mutants were expressed with NR1 in HEK293 cells and assayed by whole-cell recording. With activation of the receptor by glutamate and glycine, only a single mutant, V557C which is located at the beginning of M1, lead to irreversible inhibition by the methanethiosulfonate derivative, MTSET. The NR2 ligand, glutamate, was insufficient on its own to induce modification of V557C by MTSET, suggesting that the change in accessibility required channel gating. The rate of MTSET modification of the homologous residue on NR1 (NR1-1a$_{L562C}$/NR2A) was much slower than V557C. We also substituted cysteine in the V557 site of mutant subunits that exhibit either enhanced or reduced desensitization. Modification by MTSET correlated with the degree of desensitization for these subunits suggesting that V557C is a sensitive detector of desensitization gating.

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## Introduction

The kinetics of NMDA receptors play an important role in shaping postsynaptic responses (Jones and Westbrook, 1996; Lester and Jahr, 1992; Qian and Johnson, 2002). Although intrinsically silent, desensitized states can have significant actions on receptor gating (Jones and Westbrook, 1996). NMDA receptor desensitization can be divided into three forms with distinct underlying mechanisms: calcium-dependent; glycine-dependent; and glycine-independent (McBain and Mayer, 1994). Depending on the kinetics, desensitization can either accelerate or prolong the duration of a synaptic response. We focus here on the glycine-independent form. NMDA receptor channels are thought to desensitize directly from the closed, agonist bound state (Colquhoun and Hawkes, 1995), thus glycine-independent desensitization represents a separate closed, bound conformation. This form of desensitization contributes to the decay of EPSCs and reduces NMDA receptor mediated responses during high frequency synaptic stimulation (Lester and Jahr, 1992). Thus understanding the conformational changes associated with desensitization is ultimately important for understanding synaptic signaling.

NMDA receptors are tetramers comprised of two glycine-binding NR1 and two glutamate binding NR2 (A-D) subunits, possibly aligned in a 1-1-2-2 order (Clements and Westbrook, 1991; Inanobe et al., 2005; Schorge and Colquhoun, 2003). In some cell types NR3 subunits can be incorporated into NMDA receptors (Chatterton et al., 2002). The topology of NMDA receptor subunits consists of a large, N-terminal extracellular domain, four hydrophobic domains (M1-M4), and a cytoplasmic C-terminal domain (Mayer and Armstrong, 2004; Wollmuth and Sobolevsky, 2004). M2 is a reentrant P loop that lines the channel. The first 400 amino acids of the N-terminal domain are homologous to the leucine/isoleucine/valine-binding

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protein (LIVBP-like domain) followed by the agonist-binding S1 domain, and a short stretch of amino acids linking the S1 and M1 domains - the pre-M1 region. Based on crystal structures of AMPA receptor subunits, ligand-binding sites of NMDA receptors operate like two lobes of a clamshell composed of the S1 domain on the N-terminal domain, and the S2 domain in the extracellular loop between M3 and M4 (Mayer and Armstrong, 2004; Wollmuth and Sobolevsky, 2004). A short segment linking M3 with the S2 domain is highly conserved among ionotropic glutamate receptors and influences channel gating (Jones et al., 2002; Kohda et al., 2000). Recent crystal structures have confirmed a clamshell arrangement for the glycine-binding pocket of the NR1 subunit (Furukawa and Gouaux, 2003). Studies using the substituted-cysteine accessibility method (SCAM) on NR1 and NR2C subunits suggest that the structure of the pore resembles an inverted potassium channel, with a large extracellular vestibule surrounding the pore (Beck et al., 1999; Kuner et al., 2003). It has also been suggested that the extracellular vestibule is involved in activation and desensitization (Sobolevsky, 1999; Sobolevsky et al., 1999). However little is known about the conformational changes that couple ligand binding to channel gating.

Kinetic models indicate that when bound by two molecules of glutamate and two molecules of glycine, NMDA receptor channels have two main options (other than unbinding): they can open or desensitize. The glycine-independent form of NMDA receptor desensitization is prominent in receptors containing the NR2A or NR2B subunit. Sobolevsky et al. (1999) proposed a physical model in which glycine-independent desensitization occurs because the channel contains a desensitization “gate” that is physically distinct from the activation “gate” (Sobolevsky et al., 1999). However, mutations in several regions of the NR2A subunit alter

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desensitization in NR1/NR2A-containing receptors. These regions include the LIVBP-like domain, the pre-M1 region, the lurcher site in the third transmembrane domain, and a methionine (Met 823) in the fourth transmembrane domain (Kohda et al., 2000; Krupp et al., 1998; Ren et al., 2003; Villarroel et al., 1998; Zheng et al., 2001). Similar results have been obtained by introducing mutations in the P-loop (Asn 598) or the lurcher site of NR1 (Chen et al., 2004; Kohda et al., 2000). The pre-M1 region of NR2A is a particularly attractive candidate for coupling ligand binding to channel gating because it links the glutamate-binding S1 domain to the channel. Studies showing that the pre-M1 region influences desensitization are consistent with this idea (Krupp et al., 1998; Sobolevsky et al., 2002; Villarroel et al., 1998).

We used substituted-cysteine mutagenesis to screen the accessibility of amino acids located in and around the pre-M1 region. Cysteine-substituted NR2A subunits were coexpressed in HEK293 cells with NR1. Whole-cell recordings were used to determine the accessibility of the substituted cysteines. Of the residues screened, MTS reagents modified two mutant NR2A subunits: $NR2A_{A548C}$ and $NR2A_{V557C}$. $NR2A_{V557C}$ modification required the presence of glutamate and glycine, thus it detected channel gating. Using modified NR2 subunits with varying amounts of desensitization, we found that the accessibility of V557C correlated with desensitization.

## Materials and Methods

**Molecular biology.** All cDNAs encoding NMDA receptor subunits were cloned in pCDNA1/amp (Invitrogen, Carlsbad, CA). Clones used were NR2A (accession no. D13211; Ishii et al., 1993) and NR1-1a (accession no. U0826; Hollmann et al., 1993). The $NR1\text{-}1a_{stop838}$

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(Krupp et al., 1998) and $NR2A_{stop844}$ (Krupp et al., 2002) truncation mutants and NR2 chimeras, $2C_0A$, AD1, and D001/AD1 have been described (Krupp et al., 1998). Point mutants were generated using gene splicing by overlap extension PCR with Pfu Polymerase (Stratagene, La Jolla, CA; Horten et al., 1989). DNAs generated by PCR were sequenced. Amino acids (aa) are numbered in accordance with (Ishii et al., 1993). Lymphocyte CD4 receptor cDNA was cloned in a JPA vector kindly provided by Dr. John Adelman (Vollum Institute).

**Cell culture and transfection.** HEK293 cells (ATCC, Manassas, VA or Invitrogen) grown in DMEM (Invitrogen), 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 3 mM kynurenic acid (Sigma, St. Louis, MO), 1% glutamine, and 1% penicillin-streptomycin (Invitrogen; 37˚C, 5% $CO_2$), were plated onto 35 mm, polylysine-coated glass cover slips 3-6 hours before transfection. Cells were transfected for 12-18 hours in the presence of kynurenic acid and DL-AP5 (1 mM, Tocris, Ballwin, MO) using the $Ca^{2+}$/phosphate method (Invitrogen) at an NR1:NR2:CD4 cDNA ratio of 4:4:1. Alternatively we used the Polyfect method (Qiagen, Valencia, CA) for 6-12 hours at an NR1:NR2:CD4 cDNA ratio of 8:8:1. We stopped transfections by replacing the media with fresh media containing AP5 and FUDR (0.2 mg/ml 5'-fluoro-2-deoxyuridine and 0.5 mg/ml uridine, Sigma). Anti-CD4 receptor-coated beads (Dynal, Oslo, Norway) were used to identify transfected cells.

**Electrophysiology.** We made whole-cell voltage-clamp recordings 12-48 hours after transfections. The recording chamber was continuously superfused at room temperature (~20˚C) with extracellular solution containing (mM): NaCl 162, KCl 2.4, HEPES 10, Glucose 10, $CaCl_2$ 1 (pH 7.2, NaOH; 325 mOsm). Patch pipetttes (2-5 MΩ) were pulled from thin-walled borosilicate glass (TW150F-6; World Precision Instruments, Sarasota, FL) and filled with (mM)

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$CsCH_4SO_3$ 115.5, HEPES 10, $MgCl_2$ 6, $Na_2ATP$ 4, phosphocreatine 20, creatine phosphokinase 50 U/ml, leupeptin 0.1, BAPTA 10, $CaCl_2$ 1 (pH 7.2, CsOH; 310-320 mOsm). Where noted, the intracellular solution contained 0.1 mM EGTA and no $CaCl_2$. Solutions were prepared with HPLC grade water. Data were acquired with an Axopatch-1C amplifier and Axograph 4.5 software (Axon Instruments, Union City, CA). Unless noted, the membrane potential was clamped at -50 mV. Currents were filtered at 2 kHz and digitized at 5 kHz. Short -10 mV voltage steps before each agonist application were used to monitor cell input resistance (400-3000 MΩ). Drugs were applied by a fast microperfusion system. Unless noted, all solutions contained 100 μM glycine and agonist applications were made in 0 mM calcium. Percent block was calculated according the following equation: (1-(peak amplitude after MTS)/(peak amplitude before MTS)) X 100. The rate constant of MTSET modification was calculated from the equation derived by Wilson and Karlin (1998): (1/modification time constant) X (1/concentration of MTSET in M). Percent desensitization was calculated by the following equation: (1-(steady state current amplitude/peak current amplitude)) X 100. Data were expressed as mean ± SEM. ANOVA and Student's t test were used as appropriate with statistical significance set at $p < 0.05$.

## Results

*Screening accessibility in NR2A*

Analysis of NR2A/NR2C chimeric mutant subunits indicates that the last four residues of the NR2A pre-M1 region, residues 553-556, influence glycine-independent desensitization (Krupp et al., 1998; Villarroel et al., 1998). We mutated residues in the pre-M1 and M1 domains to cysteines to examine their accessibility during channel gating (Figure 1A). The mutants were

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co-expressed in HEK293 cells with a C-terminal truncation mutant of NR1-1a (NR1-1a$_{stop838}$), which, unless noted, we will refer to as NR1. This NR1 truncation eliminates calcium-dependent inactivation thus simplifying analysis of desensitization (Figure 1A; Krupp et al., 1999). We examined the accessibility of each mutated residue using whole-cell recordings of NMDA receptor currents. Test pulses of glutamate and glycine were delivered before and after three 5-second applications of glycine with MTSET, or glutamate and glycine with MTSET (Figure 1B). MTSET had no effect on NR1/NR2A receptors in the absence of glutamate, indicating that modification of endogenous cysteines does not alter channel gating. However MTSET caused a rapid, but completely reversible, inhibition of NR1/NR2A currents (not shown). This was attributable to open channel block by the charged MTSET rather than cysteine modification and thus did not interfere with analysis of the mutants.

Of the twelve residues we analyzed, cysteine-substitution altered the control responses of receptors containing NR2A$_{F549C}$, NR2A$_{L550C}$, NR2A$_{F553C}$, or NR2A$_{W558C}$ subunits. These currents were very small or had abnormal kinetics, and thus they were not further analyzed. Of the remaining eight residues, only a single residue, V557C, was modified by MTSET during gating. As shown in Figure 1B, NR1/NR2A$_{V557C}$ currents showed irreversible inhibition of NMDA receptor inward current following 3 applications of MTSET plus glutamate and glycine (% Block, 48 ± 3.1%, n = 19). The fast decaying peak during MTSET applications was due to reversible block of open channels as also seen with NR1/NR2A channels. The tail current that immediately followed the end of each MTSET application represented unblock of open channels. NR1/NR2A$_{A548C}$ currents were irreversibly inhibited by MTSET alone (78.4 ± 6.8%, n = 7, in the presence of glycine), indicating that the accessibility of A548C was not linked to channel

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gating. We subsequently focused our experiments on the modification of V557C during channel gating.

Substitution of MTSET with MTSEA, a smaller positively charged MTS reagent, or MTSHE, a smaller uncharged MTS reagent, only slightly increased the irreversible inhibition of NR1/NR2A$_{V557C}$ currents (61.98 ± 3.9%, n = 7 & 56.76 ± 2.4%, n = 5), suggesting that the size and charge of MTSET did not impede accessibility (Karlin and Akabas, 1998). To examine whether glutamate binding alone could alter the accessibility of V557C, we blocked the glycine-site on NR1 with 7-chlorokynurenic acid thus preventing channel activation (Figure 2). Under these conditions, glutamate application in the presence of MTSET did not cause a significant irreversible inhibition of NR1/NR2A$_{V557C}$ currents (14.9 ± 4.2%, n = 10).

*Modification rates of V557C and its homologous residue in NR1*

We determined the MTSET modification rate of NR2A$_{V557C}$ by recording NR1/ NR2A$_{V557C}$ currents before and after single glutamate, glycine, and MTSET applications of increasing durations (5-60 seconds, Figure 3). Irreversible MTSET inhibition increased exponentially, reaching 88.3 ± 2.7% block after 60 seconds. The MTSET inhibition curve was fitted with a single exponential resulting in a time constant of 18.6 seconds that corresponds to a rate constant of 26.88 $s^{-1}M^{-1}$ (Wilson and Karlin, 1998). A 15-second application of MTSET blocked a similar amount of current (53.86 ± 7.2%, n = 7) as three 5-second applications. Thus the accessibility of the V557C residue had reached equilibrium during the 5-second applications (Horn, 1998).

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Using SCAM analysis of the NR1 subunit expressed with a C-terminally truncated NR2C subunit containing an NR2A M1 domain ($NR2C^{M1}$), Beck et al. (1999) reported that leucine 562 (leucine 544 in their nomenclature) on NR1-1a was modified in the presence of glutamate and glycine (~ 67 %, 2 min, 3 mM MTSET). Leucine 562 on NR1-1a is homologous to valine 557 on NR2A. However MTSET did not irreversibly inhibit NR1-$1a_{L562C}$/NR2A currents in the presence of glutamate and glycine using the protocol shown in Figure 1. As shown in Figure 3C, NR1-$1a_{L562C}$/NR2A currents were only modified after long applications of glutamate, glycine and MTSET (40 ± 3%, 2 min, 2 mM MTSET, n = 6). Thus the accessibility of L562C on NR1-1a appeared to be slightly slower when expressed with NR2A subunits than with $NR2C^{M1}$ (Beck et al., 1999). Applications longer that 2 minutes can be damaging to cells, thus we did not attempt to determine a modification rate for the L562C mutant.

*Desensitization and accessibility of V557C*

Krupp et al. (1998) reported a series of modified NR2 constructs that demonstrate enhanced or reduced NMDA receptor desensitization. We introduced the V557C mutation into these constructs and tested for MTSET modification. $NR2C_0A_{V557C}$ and NR2D001/$AD1_{V557C}$ are NR2C/NR2A chimeras in which all or most of the N-terminus sequence is from NR2C, whereas the rest of the sequence is taken from NR2A (Krupp et al., 1998). These subunits, when expressed with NR1-$1a_{stop838}$, did not desensitize and were not irreversibly inhibited by MTSET (Figure 4). Conversely, the truncation mutant, $NR2A_{stop844V557C}$, which showed significant desensitization, had more irreversible inhibition than full-length NR2A. These results suggested that there was a correlation between desensitization and V557C accessibility. However,

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NR2AD1$_{V557C}$, which has three mutations, F553Y, A555P and S556A, that convert the pre-M1 region to the NR2C sequence, appeared to behave anomalously. It desensitized, but was not irreversibly blocked by MTSET. This likely reflects modification of desensitization by domains other than pre-M1 (Krupp et al., 1998; Villarroel et al., 1998).

*Modifying desensitization and accessibility*

If V557C accessibility occurs only when the channel is desensitized, then preventing or enhancing desensitization should alter modification by MTSET. We took two approaches to address this prediction. Tetrapentylammonium (TPentA) and 9–aminoacridine (9-AA) block open NMDA channels (Benveniste and Mayer, 1995; Costa and Albuquerque, 1994; Sobolevsky, 1999; Sobolevsky et al., 1999), and thus have been reported to block entry into desensitized states. We tested whether their presence during channel gating might prevent MTSET modification of NR1/NR2A$_{V557C}$ receptors. Co-application of TPentA (1 mM) with MTSHE, glutamate, and glycine did not reduce irreversible inhibition of NR1/NR2A$_{V557C}$ currents (47.7 ± 10.5%, n = 5). However TPentA only slightly prevented desensitization (36.1 ± 2.2% desensitization in control compared to 21.6 ± 4% desensitization in TPentA, n = 7). 9-aminoacridine (100 μ□) reduced irreversible MTSET inhibition in two cells (24% and 22%). However, the effect of 9-AA was overcome when we used 10 mM MTSET (66.3 ± 5.6%, n = 4). If one assumes that 9-AA prevents desensitization, then the result with 10 mM MTSET suggests that 9-AA and MTSET may compete for binding within the pore.

Another way to alter desensitization is by altering intracellular calcium. Influx of calcium through NMDA receptors increases desensitization as a result of calcineurin (CaN) activation

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(Krupp et al., 2002). Thus we tested if enhancing desensitization with calcium influx would increase the accessibility of V557C. Following 5-second applications of glutamate, glycine, and calcium (2 mM) to activate CaN, we measured MTSET modification of V557C in calcium-free solutions (Figure 5). Calcium influx increased the desensitization and the irreversible inhibition of currents mediated by NR1/NR2A$_{V557C}$. Furthermore, calcium influx induced modification of NR1/NR2AD1$_{V557C}$ receptors. This protocol did not affect the accessibility of the homologous L562C residue in NR1. Calcium influx significantly increased desensitization of NR1-1a$_{L562C}$/NR2A but there was no detectable irreversible inhibition (72.3 ± 3.7% desensitization, 8.8 ± 7.8% MTSET block, n = 4, data not shown).

The correlation between desensitization and the modification of V557C for the NR2A constructs is plotted in Figure 6 ($R = 0.86$, $p < 0.01$).

## Discussion

*Validity of method and comparison to prior results*

The substituted-cysteine accessibility method has been used to infer conformational movements of several ion channels and ligand-gated receptors (Karlin and Akabas, 1998). The general assumptions of the method are that cysteine substitution does not alter channel properties; that any changes in channel properties reflect modification of the substituted cysteine rather than native cysteines; and that the sufhydryl modification is irreversible. It should also be noted that MTS reagents could modify substituted cysteines without altering receptor function, thus escaping detection by SCAM. In many cases, these studies have been directed at the relatively constrained environment of the channel pore. Within the pore, the validity of the

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SCAM assumptions is relatively easy to establish. Changes in cysteine accessibility with channel activation have also been used to define residues involved in channel gating (Liu et al., 1996; Yang and Horn, 1995). We applied this method to desensitization of NMDA receptors. Given the location of the pre-M1 region near the NMDA receptor extracellular vestibule and its importance in glycine-independent desensitization, we used SCAM to examine twelve residues in pre-M1 and the first transmembrane domain of NR2A. Four cysteine mutants yielded nonfunctional or abnormal currents when co-expressed with NR1-1a$_{stop838}$. The eight other cysteine mutants had normal current amplitudes and kinetics suggesting that cysteine substitution did not significantly alter their secondary structure. Although MTSET blocked open NMDA channels, this effect was reversible and thus did not represent modification of native or substituted-cysteine residues.

SCAM has been used to define the structure of the pore and the extracellular vestibule of the NMDA receptor channel. In these studies, the number of modified residues in the pore was sensitive to the size of MTS reagents (Kuner et al., 1996). This indicated that the pore is a narrow structure formed by M2 domains centered on the magnesium-binding site. M3 domains occupy most of the extracellular vestibule whereas portions of pre-M1, M1, and M4 contribute to the vestibule. MTS reagents modify many residues in the vestibule in the absence of channel activation (Beck et al., 1999). In our experiments, MTSET irreversibly inhibited currents mediated by receptors containing NR2A$_{A548C}$ or NR2A$_{V557C}$, but only modification of NR2A$_{V557C}$ required channel gating. Both glutamate and the co-agonist glycine were required for modification of V557C, suggesting that the change in accessibility involved a concerted action of NR1 and NR2 subunits, rather than a direct effect of glutamate binding to the NR2 subunit.

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The changes in accessibility of V557C with gating are unique within the preM1-M1 region. Previous studies reported that the homologous residue in NR1 also is modified by MTSET (Beck et al., 1999). In those experiments NR1-1a$_{L562C}$ (L544 in the terminology of Beck et al.) was expressed with a modified NR2C subunit in *Xenopus* oocytes. When we expressed the same construct with wild-type NR2A, the modification was less. This result suggests that NR2 subunits influence L562C accessibility on NR1. The different rates of accessibility for V557C on NR2 and L562C on NR1 may also reflect asymmetrical gating of NR1 and NR2 subunits (Wollmuth and Sobolevsky, 2004) or simply differences in the specific environments of the cyteines in NR1$_{L562C}$ and NR2$_{V557C}$ subunits.

*Channel blockers and NMDA channel gating*

Using channel blockers of varying sizes, Sobolevsky et al. (1999) reported that small blockers, such as tetraethylammonium (TEA), were trapped in the open channel without affecting channel closure or desensitization, whereas a large blocker, tetrapentylammonium (TPentA), prohibited channel closure as well as desensitization. On this basis they proposed a physical model of NMDA receptor channel gating that contains distinct activation and desensitization “gates” with the activation gate placed closer to the extracellular surface. This idea was based on the fact that the intermediate size blocker, tetrabutylammonium (TBA), prevented desensitization, but not channel closure. However, placement of the activation gate near the extracellular surface is incompatible with the fact that many residues in the vestibule are accessible to MTS reagents in the absence of agonist (Beck et al., 1999). The SCAM analysis of non-desensitizing NR1/NR2C receptors suggests that the activation gate is deep within the pore

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formed by M3 segments whereas the pre-M1 and M4 segments form the more superficial lining of the extracellular vestibule. In this model, channel closure involves constriction of the deep part of the vestibule, thus trapping certain channel blockers (Sobolevsky et al., 2002).

This revised model leaves open the possibility that residues involved in desensitization might be located in the superficial parts of the vestibule. We expected that channel blockers that purportedly block desensitization would be useful to examine changes in the accessibility of residues in the pre-M1/M1 region. Unfortunately, TPentA did not completely block desensitization in our experiments, and the effects of 9-aminoacridine were not interpretable due to its competition with MTSET, itself an NMDA channel blocker. Thus the use of channel blockers was not sufficient in our hands to define the domains involved in NMDA receptor desensitization.

*Modification of V557C and desensitization*

Different combinations of NMDA receptor subunits have striking differences in desensitization. The amino acid sequence differences between desensitizing NR2A subunits and nondesensitizing NR2C subunits were used to show that the pre-M1 region and the LIVBP-like domain in the N-terminus are involved in NMDA receptor desensitization (Krupp et al., 1998; Villarroel et al., 1998). Our results provide further evidence that residues in the pre-M1 region are sensitive to gating steps associated with desensitization. The overall correlation between desensitization and MTSET modification suggests that V557C, the first residue in M1, was only modified when the receptor was in the desensitized state. This correlation was supported by the increases in MTSET modification resulting from calcium-induced increases in desensitization of

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NR1/NR2A$_{V557C}$ currents. These last results also support the idea that intracellular regulation of NR2A modifies desensitization at a distance by affecting regions at, or surrounding, V557C. This action of the intracellular domain of NR2A, possibly influenced by an intracellular protein-protein interaction, could alter the conformation of the N-terminal domain (Krupp et al., 2002).

In addition to the pre-M1 region, the LIVBP-like domain also influences desensitization. Thus it is reasonable to consider whether the LIVBP-like domain could explain the apparently anomalous behavior of NR2AD1$_{V557C}$. This mutant is identical to NR2A except for an NR2C pre-M1 region. The LIVBP-like domain affects glycine-independent desensitization through an allosteric effect (Erreger and Traynelis, 2005; Zheng et al., 2001), yet desensitization of NR1/NR2AD1 receptors persists in the absence of zinc (Hu and Zheng, 2005). We did not test the mutant in the presence of zinc chelators, thus further experiments will be necessary to fully understand desensitization of NR1/NR2AD1$_{V557C}$ receptors. However, when desensitization in this mutant was enhanced by CaN activation, the receptor was modified by MTSET. Thus accessibility of V557C is a reliable detector of desensitization.

*A structural view of desensitization gating*

Our results indicate that modification of V557C correlates with NMDA receptor desensitization. The proximity of valine 557 to the pre-M1 region fits with a critical role of that region in desensitization, but, as discussed above, other domains including the LIVBP-like domain and regulatory effects in the C-terminus also contribute to desensitization. Models based primarily on studies of the channel pore cannot account for these more distant effects (Sobolevsky et al., 1999), nor can they account for the coupling between ligand binding and

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channel gating. Thus, it is necessary to incorporate a model of conformational changes associated with ligand binding to understand desensitization. Such information largely comes from studies of AMPA receptors.

The crystal structures of the ligand-binding pocket of AMPA receptors provide a framework to consider how ligand binding leads to channel gating including desensitization. Structures with and without ligands have provided information on the conformational movements triggered by ligand binding, that have been tested in functional studies (Mayer and Armstrong, 2004; Sun et al., 2002). These studies indicate that movements in ligand-binding domains directly cause changes in gating. Several basic features emerge that are likely shared among ionotropic glutamate receptors. Each AMPA receptor is a tetramer comprised of two subunit pairs. The subunits within each pair non-covalently interact with each other through their S1 domains. Glutamate initially binds to the S1 pocket of an open S1-S2 clamshell causing displacement of S2 away from the plasma membrane as it closes around the ligand. This S2 movement causes channel gating by pulling the M3 domains up and away from each other. The open channel confirmation puts tension on the interactions between a pair of S1 domains. Desensitization occurs when S1-S1 interactions rearrange into a more stable confirmation causing transmembrane domains to relax and close the channel (Horning and Mayer, 2004).

Although there is less structural information on NMDA receptor subunits, there are reasons to expect similarities with AMPA receptors. NMDA and AMPA receptors share similar kinetic schemes; they have very similar secondary structures; the crystal structure of the NR1 ligand-binding domain is similar to AMPA subunits; NR2A requires a portion of the LIVBP-like domain near the S1 of NR1 for surface expression; and S1 domains mediate negative

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cooperativity between NR1 and NR2 subunits (Furukawa and Gouaux, 2003; Inanobe et al., 2005; Meddows et al., 2001; Regalado et al., 2001; Wollmuth and Sobolevsky, 2004). Assuming that NMDA receptors are NR1/NR2 dimers of dimers arranged in 1-1-2-2 tetramers, (Schorge and Colquhoun, 2003), desensitization may involve a relaxation in S1-S1 interfaces. Our results suggest that this relaxation is sensed by the linker connecting S1 to M1, resulting in the accessibility of V557C in NR2A (the glutamate binding subunit). Interestingly, the homologous NR1 residue was actually more accessible in a non-desensitizing receptor (Beck et al., 1999). The change in accessibility of V557C is consistent with a specific role of the pre-M1 region in NMDA receptor desensitization. Our data cannot resolve whether the putative relaxation of the S1-S1 interface simply reveals V557C, making it a sensitive detector of the desensitized state, or if V557C is the desensitization "gate". Even if the latter is true, our data confirm that the LIVBP-like domain can independently influence desensitization and thus presumably the S1-S1 relaxation. The modulation by CaN further suggests that intracellular domains can induce conformational changes in ligand-binding domains.



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**Figure Legends**

Figure 1. MTSET modification of $NR2A_{V557C}$ occurred in a state-dependent manner. A: Diagram of the NR2A subunit. Residues around the pre-M1/M1 border (enlarged) were individually mutated to cysteine (bold lettering). Valine 557 is underlined. B: MTSET modification screening protocols. Top and bottom recordings are from two different HEK293 cells expressing the NR1-$1a_{stop838}$/$NR2A_{V557C}$ subunit combination. Test pulses of glutamate (1 mM) and glycine (100 μM) were recorded before (extreme left) and after (extreme right) three applications of glutamate, glycine, and MTSET (2 mM, top traces) or glycine and MTSET (bottom traces). The test pulses are averages of 3-4 responses. C: The irreversible inhibition by MTSET was plotted for each cysteine-substituted NR2A subunit using the protocol in panel B. Data represents mean ± SEM (* = significant compared to wild type NR2A; ANOVA with Bonferroni/Dunn post-hoc test $p < 0.0005$; ** = significantly different than % block for V557C tested with glycine and MTSET, unpaired *t*-test $p < 0.0001$).

Figure 2. MTSET modification of $NR2A_{V557C}$ requires channel gating. Currents mediated by NR1-$1a_{stop838}$/$NR2A_{V557C}$ receptors were recorded in the presence of glutamate and glycine immediately before and after three applications of glutamate (1 mM), 7-chlorokynurenic acid (100 μM, 7CKA), and MTSET (2 mM). The glycine antagonist, 7CKA, completely blocked the evoked current as well as glutamate- and glycine-dependent modification by MTSET. The irreversible inhibition after three applications of 7CKA and MTSET (8.8 ± 2.7%, n = 6; unpaired *t*-test) was the same as for glutamate, 7CKA and MTSET (14.9 ± 4.2%, n = 10). The small reduction was caused by gradual rundown of current amplitudes.

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Figure 3. MTSET modification rates for $NR2A_{V557C}$ and NR1-$1a_{L562C}$. A: The sequences of the pre-M1 and M1 regions of NR2A and NR1 are shown. B: Example responses from cells transfected with NR1-$1a_{stop838}$/$NR2A_{V557C}$ (top) and NR1-$1a_{L562C}$/NR2A (bottom). Test pulses were recorded before (left-hand traces) and after (right-hand traces) a 60-second application of glutamate, glycine, and MTSET (2 mM, middle traces). Test pulses are averages of 3-4 traces. C: MTSET modification rates for each clone, derived from the % irreversible block observed after different MTSET application lengths. Each point is the average of at least 6 cells. The NR1-$1a_{stop838}$/$NR2A_{V557C}$ averages were fit with a single exponential. MTSET inhibition of NR1-$1a_{stop838}$/$NR2A_{V557C}$ responses recorded after 30, 45, and 60-second applications of glutamate, glycine, MTSET were not significantly different from each other (ANOVA and a Bonferroni/Dunn post-hoc test). For NR1-$1a_{L562C}$/NR2A responses, only a 120-second application of glutamate, glycine, and MTSET caused significant irreversible inhibition ($p < 0.0001$).

Figure 4. MTSET modification of $NR2A_{V557C}$ correlates with increased desensitization. A, left-hand column: Diagrams of NR2A/NR2C chimeric, NR2A wild-type, and NR2A truncated subunits containing the V557C mutation. Black bars represent NR2C sequence and white bars represent NR2A sequence. Middle and left-hand columns are representative test pulse responses from each construct, expressed with NR1-$1a_{stop838}$. Each was recorded before (middle column) and after (right-hand column) three, 5-second applications of glutamate, glycine, and MTSET. B: Summary of average % desensitization and % MTSET irreversible inhibition (% Block).

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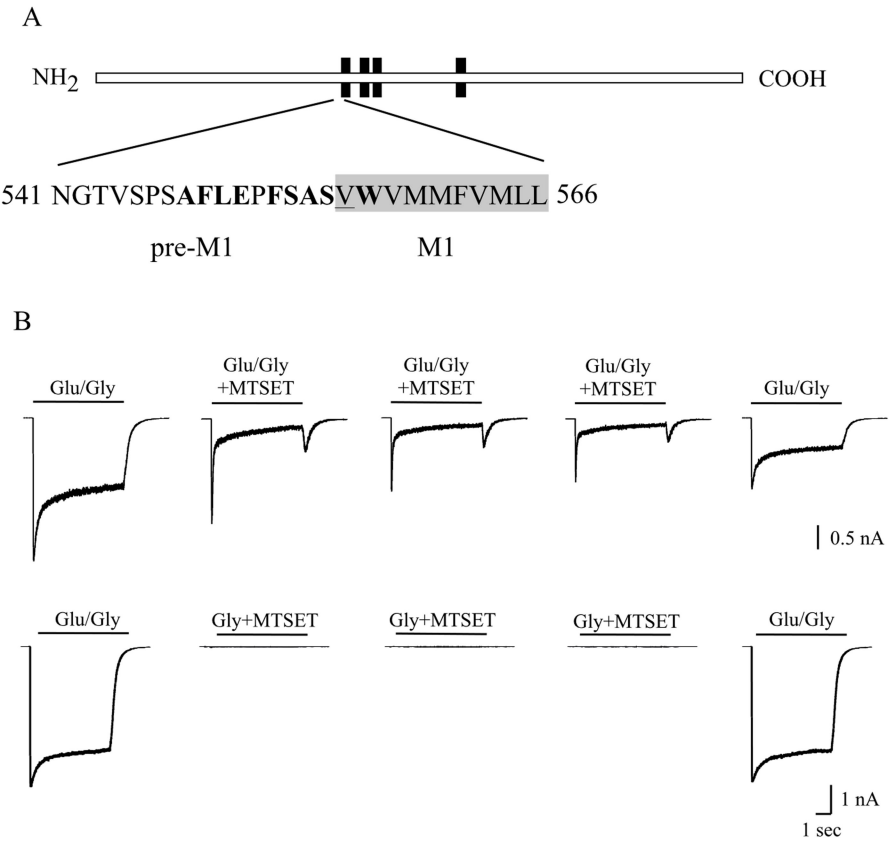
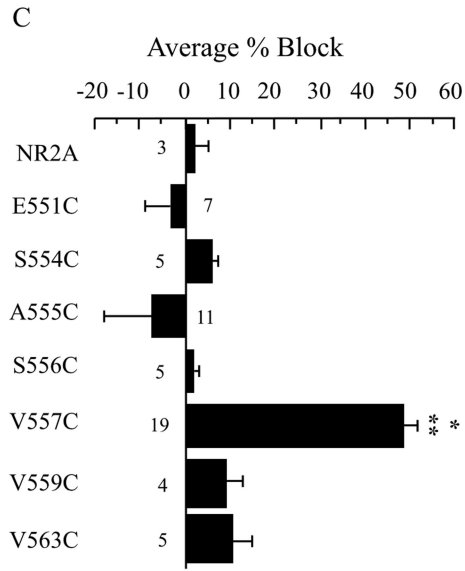
ANOVA with a Bonferroni/Dunn post-hoc test was used to analyze differences ($NR2C0A_{V557C}$, n = 3; $NR2D001/AD1_{V557C}$, n = 7; $NR2AD1_{V557C}$, n = 5; $NR2A_{V557C}$, n = 19; $NR2A_{stop844V557C}$, n = 7; * = significantly different than $NR2A_{V557C}$, $p < 0.0001$ for % desensitization; $p < 0.0005$ for % block).

Figure 5. Calcium influx increases desensitization and MTSET irreversible inhibition in $NR2A_{V557C}$- and $NR2AD1_{V557C}$- containing receptors. A: Examples of recordings from NR1-$1a_{stop838}$/$NR2A_{v557c}$- and NR1-$1a_{stop838}$/$NR2AD1_{v557c}$- transfected cells (top and bottom traces, respectively). Initial glutamate and glycine test pulses (left) showed some desensitization. Subsequent glutamate and glycine applications in the presence of calcium (2 mM, middle traces) decreased the current amplitude and increased desensitization. Test pulses recorded again in calcium-free conditions before and after applying glutamate, glycine, and MTSET three times (right-hand traces). B: The % desensitization and % MTSET irreversible inhibition (% Block) were measured after calcium treatment ($Ca^{2+}$Tx) for each clone. Unpaired *t*-tests were used to compare $NR2A_{V557C}$ with $NR2A_{V557C}$ after $Ca^{2+}$Tx averages (*, $p < 0.0001$ for % block) and $NR2AD1_{V557C}$ with $NR2AD1_{V557C}$ after $Ca^{2+}$Tx averages (**, $p < 0.02$ for % block and $p < 0.01$ for % desensitization).

Figure 6. MTSET modification correlates with desensitization. Data from Figures 4 and 5 were combined and plotted as a correlation. A line fit of the data yielded a correlation coefficient of 0.86, $p < 0.01$.

Figure 1


A
NH2
COOH
541 NGTVSPSAFLEPFSASVWVMMFVMLL 566
pre-M1
M1
B
Glu/Gly
Glu/Gly +MTSET
Glu/Gly +MTSET
Glu/Gly +MTSET
Glu/Gly
0.5 nA
Glu/Gly
Gly+MTSET
Gly+MTSET
Gly+MTSET
Glu/Gly
1 nA
1 sec


C
Average % Block
-20 -10 0 10 20 30 40 50 60
NR2A 3
E551C 7
S554C 5
A555C 11
S556C 5
V557C 19
V559C 4
V563C 5
* * *

# Figure 2

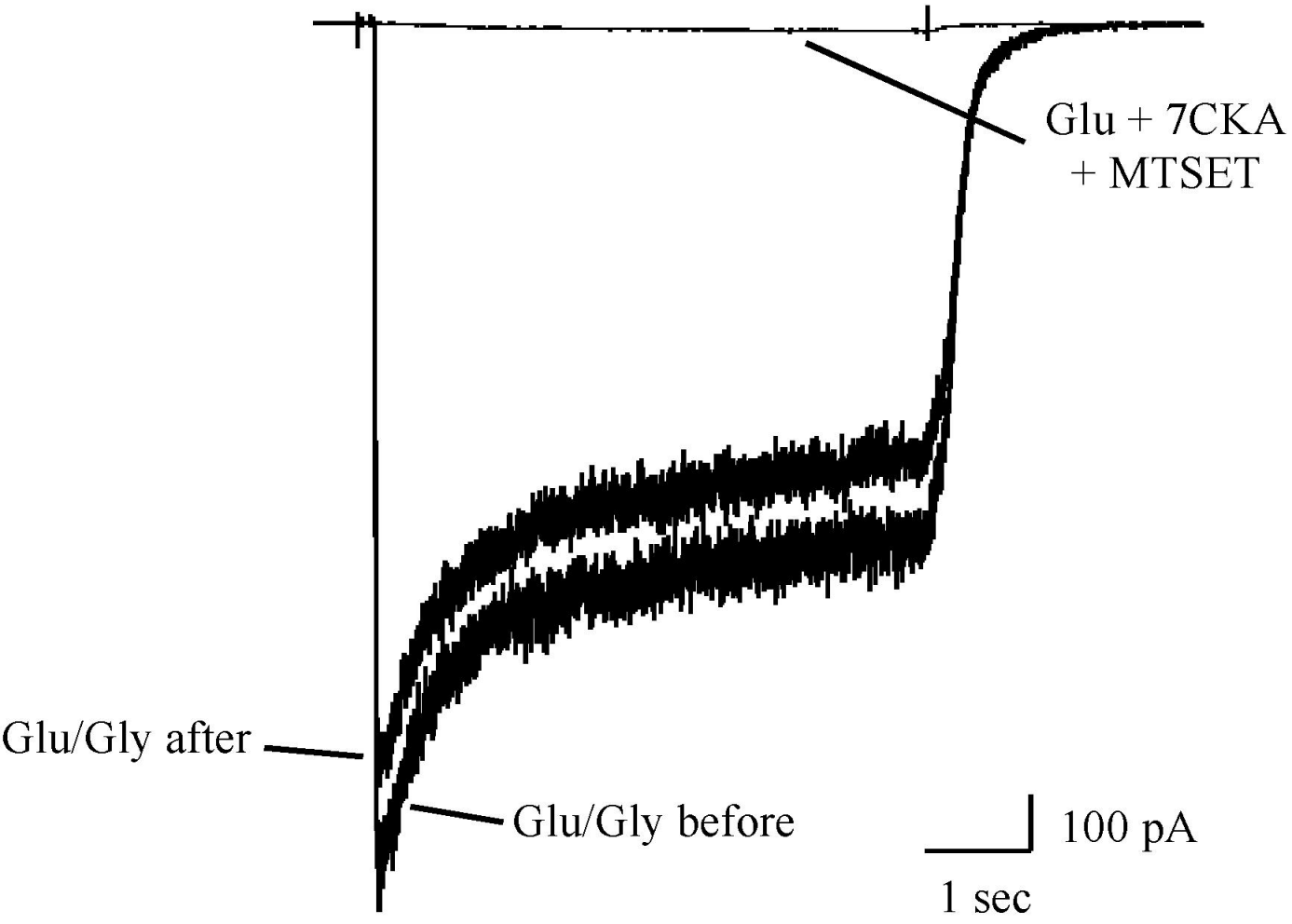
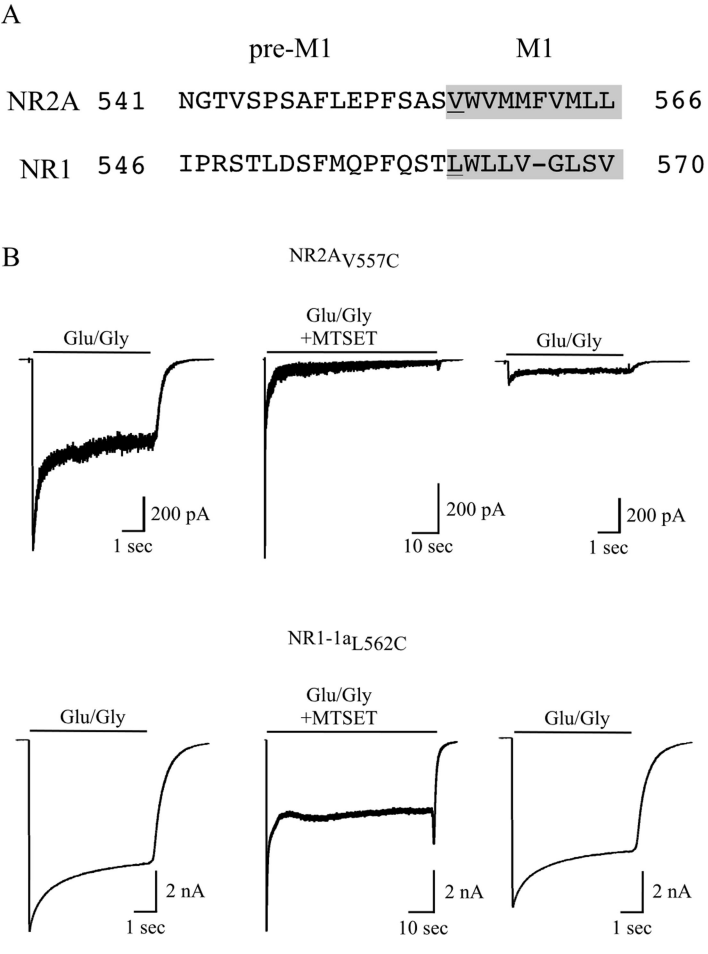


Figure 3


A
pre-M1
M1
NR2A 541 NGTVSPSAFLEPFSASVWVMMFVMLL 566
NR1 546 IPRSTLDSFMQPFQSTLWLLV-GLSV 570
B
NR2A V557C
Glu/Gly
Glu/Gly +MTSET
Glu/Gly
200 pA
1 sec
200 pA
10 sec
200 pA
1 sec
NR1-1a L562C
Glu/Gly
Glu/Gly +MTSET
Glu/Gly
2 nA
1 sec
2 nA
10 sec
2 nA
1 sec

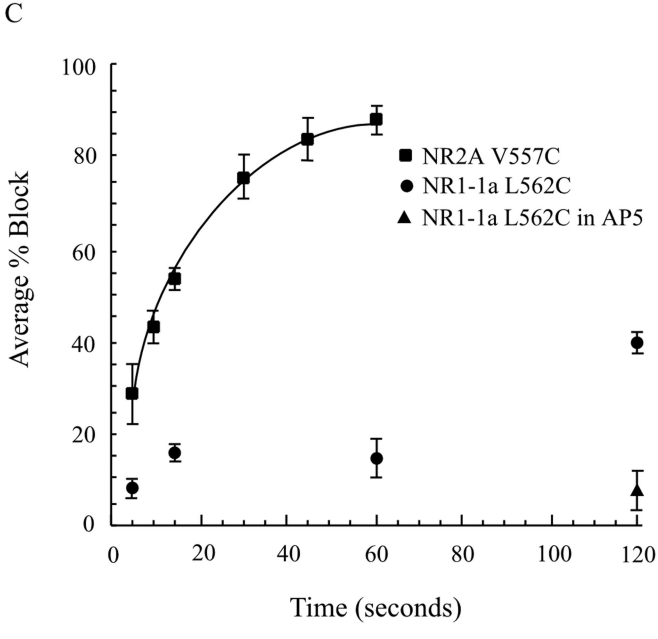

C
Average % Block
Time (seconds)
NR2A V557C
NR1-1a L562C
NR1-1a L562C in AP5
0
20
40
60
80
100
120

Figure 4

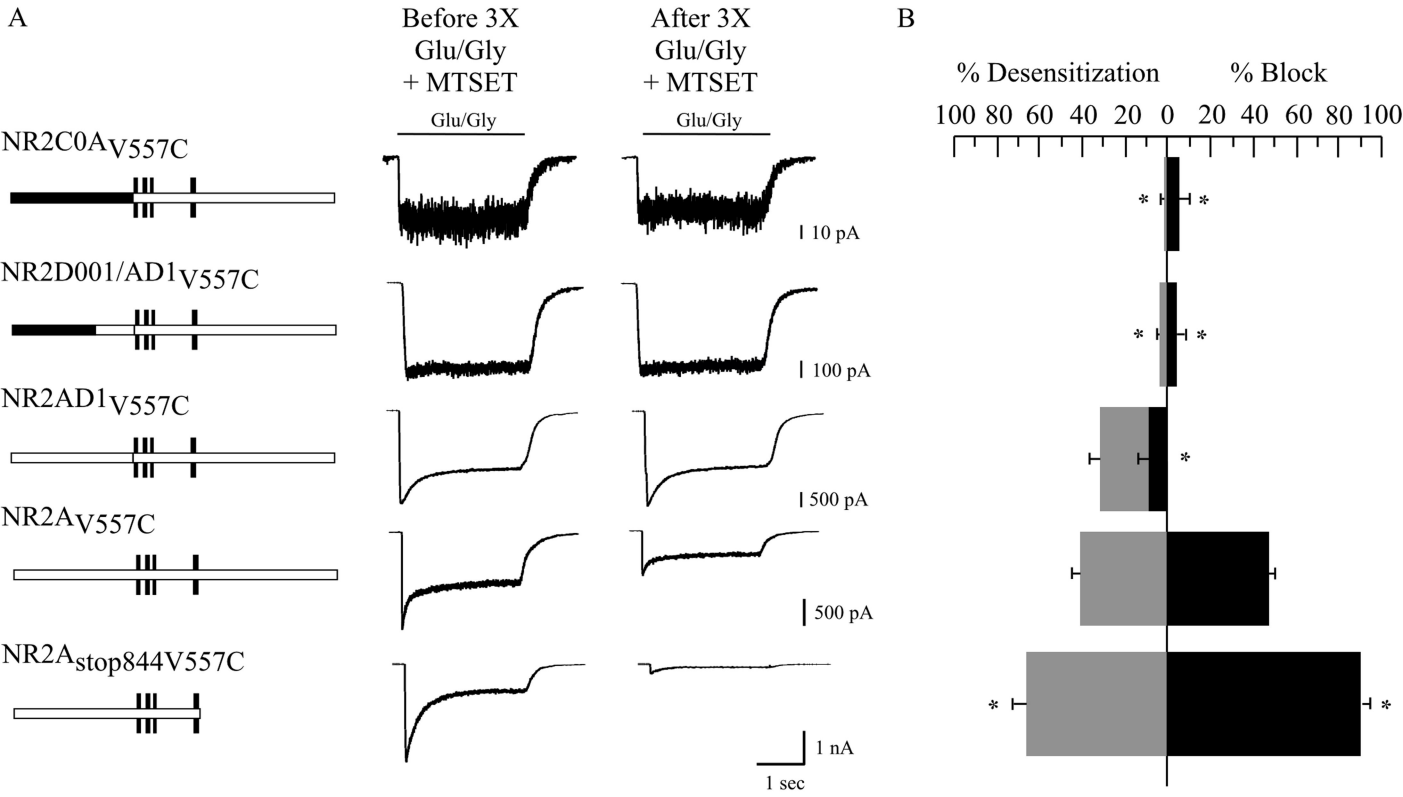
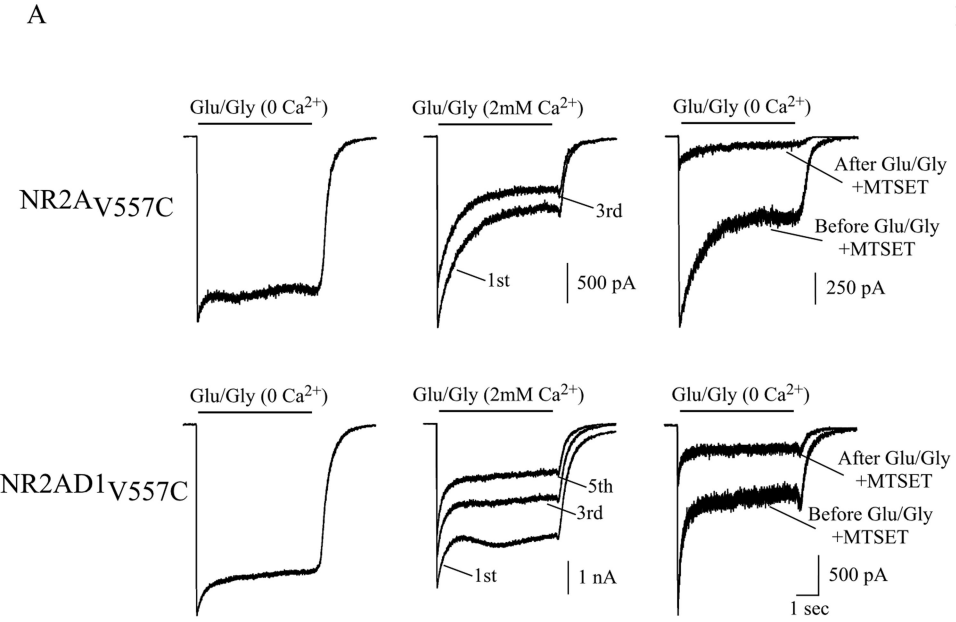
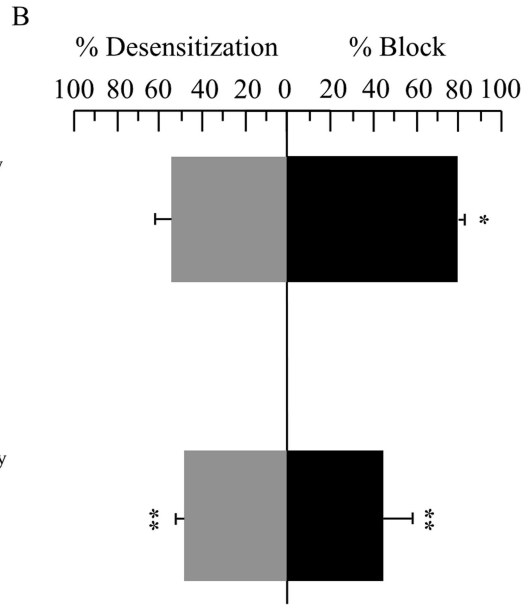
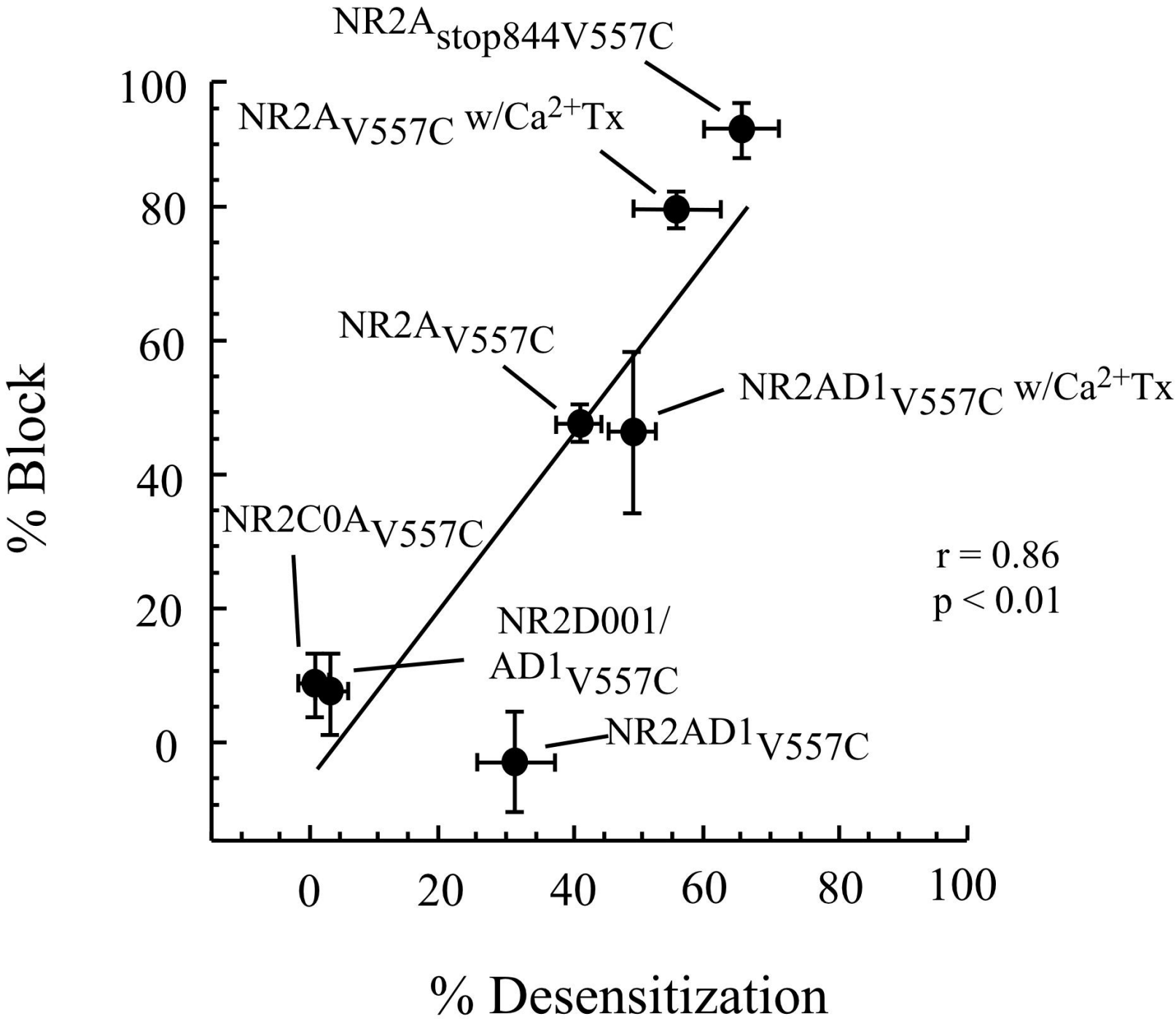

A
Before 3X Glu/Gly + MTSET
After 3X Glu/Gly + MTSET
Glu/Gly
Glu/Gly
NR2C0A V557C
NR2D001/AD1 V557C
NR2AD1 V557C
NR2A V557C
NR2A stop844V557C
10 pA
100 pA
500 pA
500 pA
1 nA
1 sec
B
% Desensitization
% Block
100 80 60 40 20 0 20 40 60 80 100

Figure 5


A
Glu/Gly (0 Ca2+)
Glu/Gly (2mM Ca2+)
Glu/Gly (0 Ca2+)
NR2AV557C
3rd
1st
500 pA
After Glu/Gly
+MTSET
Before Glu/Gly
+MTSET
250 pA
Glu/Gly (0 Ca2+)
Glu/Gly (2mM Ca2+)
Glu/Gly (0 Ca2+)
NR2AD1V557C
5th
3rd
1st
1 nA
After Glu/Gly
+MTSET
Before Glu/Gly
+MTSET
500 pA
1 sec


B
% Desensitization
% Block
100 80 60 40 20 0 20 40 60 80 100
*
**
**

# Figure 6


NR2A stop844V557C
NR2A V557C w/Ca2+Tx
NR2A V557C
NR2AD1 V557C w/Ca2+Tx
NR2C0A V557C
NR2D001/
AD1 V557C
NR2AD1 V557C
r = 0.86
p < 0.01
% Block
% Desensitization
0
20
40
60
80
100