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Molecular Determinants of Glycine-independent Desensitization of NR1/NR2A Receptors

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List of non-standard abbreviations:

NMDA, N-methyl-D-aspartate

ATD, amino-terminal domain

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ABSTRACT

Glycine-independent desensitization is thought to be an important regulatory mechanism for the function of NMDA receptors. Previous studies have suggested that the molecular determinants for glycine-independent desensitization are located at two distinct domains of NR2A, i.e. the amino-terminal domain (ATD) and the pre-M1 domain. Since the glycine-independent desensitization described in these earlier studies were a mixture of glycine-independent desensitization and zinc-dependent apparent desensitization, the exact role of these two domains in glycine-independent desensitization remains in question. In the present study, we show that deletion of the ATD of NR2A or mutating the pre-M1 region of NR2A causes no detectable changes in the degree or the time constant of glycine-independent desensitization. Therefore, the ATD and the pre-M1 domain of NR2A play no significant role in glycine-independent desensitization of NR1/NR2A receptors. On the other hand, several residues in the *lurcher* motif of either NR1 or NR2A are critical for the glycine-independent desensitization of NR1/NR2A receptors. In addition to NR1a(A653T) and NR2A(A651T), NR1a(T648C), NR1a(A649C), NR2A(T646C) and NR2A(A647C) show significantly reduced glycine-independent desensitization. Since all these mutations also alter the proton sensitivity or deactivation time constants of NR1/NR2A receptors, our data suggest that the channel gating and desensitization of NMDA receptors share common molecular determinants and the *lurcher* motif of NR1 and NR2A is critical for both processes.

INTRODUCTION

Excitatory amino acids, such as glutamate, are the most widely used neurotransmitter in the vertebrate central nervous system. The NMDA receptor, a subtype of glutamate receptor exhibiting high calcium permeability, plays a critical role in synaptic plasticity and neuronal development (for review, see Dingledine et al., 1999). Hyperactivity of NMDA receptors, causing calcium overload in neurons, are implicated in a variety of pathological conditions, such as stroke (Dirnagle et al., 1999; Lee et al., 1999), head trauma (Obrenovitch and Urenjak, 1997), limbic seizures (Meldrum et al., 1999), and degenerative neurological diseases (Olney et al., 1997, 2001). Hypoactivity of NMDA receptors has been suggested to be an underlying cause for schizophrenia (Coyle et al., 2003).

Alteration of the time course of macroscopic NMDA receptor currents has important functional implications since it can drastically change the amount of calcium influx. One mechanism by which the time course of macroscopic currents can be modulated is desensitization, broadly defined as a reduction of macroscopic NMDA currents in the continuous presence of glutamate. Several forms of "desensitization" of NMDA receptors as broadly defined have been reported (for details, see Zheng et al., 2001). The most prominent form of desensitization is the "glycine-independent desensitization", defined as desensitization of NMDA receptors that are calcium-independent and cannot be prevented by a high concentration of glycine (Sather et al., 1990, 1992).

The molecular determinants of glycine-independent desensitization have been investigated recently. It has been suggested that the amino-terminal domain (ATD) and several amino acid residues leading to the first transmembrane domain (the pre-M1 region) of NR2A are critical for glycine-independent desensitization (Krupp et al., 1998; Villaroel et al., 1998). The two

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components of glycine-independent desensitization of NR1/NR2A receptors appear to correspond to these two regions. Mutations of the pre-M1 region selectively eliminate the slow component of glycine-independent desensitization (τ=2 sec) while substitution of the ATD of NR2A with the corresponding region of NR2C selectively eliminates the fast component of glycine-independent desensitization (τ=0.3 sec). In addition to these domains in NR2A, the NR1 subunit may also play a role in desensitization of NMDA receptors. A single point mutation (NR1a(A653T)) of NR1 has been reported to alter desensitization of NMDA receptors (Khoda et al., 2000). This point mutation is frequently referred to as the *lurcher* mutation since it is initially identified in the δ2 glutamate receptors of the *lurcher* mice (Zuo et al., 1997). It is located in a short stretch of highly conserved amino acid residues known as the *lurcher* motif.

The fast component of glycine-independent desensitization in earlier reports is actually caused by ambient zinc, acting at the extracellular high affinity zinc site (Zheng et al., 2001). Since the desensitization reported in those earlier studies are a mixture of the fast and the slow desensitization, it is uncertain whether these domains reported previously truly play a critical role in glycine-independent desensitization. The reduction in the degree of overall desensitization could be accounted for by a change in the zinc-dependent apparent desensitization. In this study, we attempted to re-evaluate the role of these domains in glycine-independent desensitization under nominally zinc-free conditions. Since the fast component of desensitization of NR1/NR2A receptors is actually caused by zinc, data from earlier studies appear to imply that the pre-M1 region is the sole determinant of the glycine-independent desensitization. We expected that mutations in the pre-M1 region should abolish the glycine-independent desensitization. However, we found that mutations in the pre-M1 region have no effects on glycine-independent desensitization. Furthermore, the deletion of the ATD of NR2A also has no

effects on the degree or time constant of glycine-independent desensitization. These findings suggest that neither the pre-M1 region nor the ATD of NR2A play a critical role in glycine-independent desensitization and prompted us to explore other regions of the NMDA receptors. We showed that the *lurcher* mutation (NR1aA653T or NR2AA651T) reduce the glycine-dependent desensitization. We also identified additional residues in the *lurcher* motif that reduce or abolish glycine-independent desensitization of NR1/NR2A receptors. Our data suggest that the *lurcher* motif is critical for glycine-independent desensitization and that the gating and desensitization of NMDA receptors may share common structural determinants.

Materials and Methods

Molecular Biology. Most plasmid encoding wild type NR1/NR2A receptors and mutants used in this study were described previously (Low et al., 2003). The ATD deletion of NR2A was constructed by shuttling the EcoR I-Xba I fragment of pRSCI-A'ΔN1-3 provided by J. Neyton (Fayyazuddin et al., 2000) into pCI-NR2A with a Xba I site introduced to the pre-M1 region. The resultant mutant lacks the first 385 amino acids of NR2A but the remaining amino acid sequences were identical to the wild type NR2A.

Transfection of HEK cells. HEK 293tsa cells (Dr. Rick Horn, Tufts University, Philadelphia, PA) or regular HEK293 cells (American Type Culture Collection, Manassas, VA) were maintained at 37° and 5% CO_2 in DMEM supplemented with L-glutamine (200 μM), sodium pyruvate (100 μM), penicillin/streptomycin (100 units/ml), and 10% fetal bovine serum (Invitrogen, Calsbad, CA). Low confluency cells were transfected by the calcium phosphate precipitation method (Chen and Okayama, 1987). Cells were cotransfected with a mixture containing NR1, NR2, and GFP plasmids (1, 2, and 0.3 μg per 12 mm diameter coverslip, respectively). After transfection, D-AP5 (100-200

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μM) and 7-Cl-kynurenic acid (50-100 μM) was added to the culture medium. For some mutants, $MgCl_2$ (2 mM) was also needed to reduce excitotoxicity. NMDA antagonists were purchased from either Sigma (St. Louis, MO) or Tocris Cookson (Ballwin, MO).

Whole cell patch-clamp recordings from HEK 293 cells. Patch clamp recording in the whole cell configuration was made as described previously using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Recording electrodes (5-12 MΩ) were filled with (in mM): 140 Cs-gluconate, 5 HEPES, 4 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 1 ATP, 0.3 GTP and 5 BAPTA (pH 7.4). The recording chamber was continually perfused with recording solution comprised of (in mM): 150 NaCl, 10 HEPES, 1.0 $CaCl_2$, 3 KCl, and 20 mM mannitol. Glutamate (100 μM) and glycine (30-60 μM) was applied using a double-barrel glass pipette driven by a piezo-based fast solution jumping system (Burleigh LSS 3100, EXFO Burleigh, Victor, NY). The rise time of the junction potential change produced by this system is 0.66 ms. The free zinc concentration was reduced to nominally "zero" nM by adding 10 μM EDTA or 10 mM tricine in all solutions. The degree of the desensitization was quantified by the ratio of the steady state current amplitude at the end of a long glutamate pulse over the peak current amplitude. To avoid complications from calcium-dependent inactivation, we only collected and analyzed outward current at positive holding potentials. To reduce the time-dependent variation of desensitization, we limited the data collection to a narrow time window of 5-20 min after establishing the whole cell patch-clamp.

Curve fitting. The time course of desensitization was fitted with one exponential component with Clampfit 9 (Axon Instruments, Union City, California) using the following equation.

$$A(t)=A_0+A\times\exp(-t/\tau_n) \quad (1)$$

Data analysis and statistics. All pooled data was expressed as mean±SEM. For comparisons of multiple mutants, we used one-way analysis of variance (ANOVA) with Tukey *post hoc* test.

Homology modeling of the pore regions of the NMDA receptor. A homology model of the pore regions of the NMDA receptor was constructed from an alignment between alternating NR1 (residues Ser553-Arg659) and NR2A (residues Phe549-Glu657) subunits and the subunits of MthK potassium channel (1LNQ; Jiang et al., 2002a, b). We assumed an arbitrary subunit arrangement of NR1-NR2-NR1-NR2. Sequences were aligned on the first transmembrane domain and the M2 reentrance loop. The alignment was submitted to the online server at the Swiss Institute of Bioinformatics (http::/www.expasy.org/swissmodel/SWISS-MODEL.html).

RESULTS

The pre-M1 region and the ATD of NR2A are not critical for glycine-independent desensitization

To isolate the glycine-independent desensitization from the fast zinc-dependent apparent desensitization, we added metal chelators (10 mM tricine or 10 μM EDTA) in all of our recording solutions. To minimize the complication from calcium-dependent inactivation, we analyzed only the outward NMDA currents recorded at a positive holding potential. The glycine-independent desensitization under these conditions can be well fitted with a single exponential component that is similar to the slow component described in earlier studies (Zheng et al., 2001).

To determine the role of the pre-M1 region in glycine-independent desensitization, we recorded the NMDA currents in HEK 293 cells co-transfected with NR1 and NR2A(AD1). NR2A(AD1) is the pre-M1 mutant of NR2A (kindly provided by Drs. S.F. Heinemann and G.L. Westbrook) in which three residues of the wild type NR2A in the pre-M1 region are mutated to corresponding residues in NR2C (Krupp et al., 1998). If the pre-M1 region is the sole structural

determinant of glycine-independent desensitization, the remaining apparent desensitization for NR1/NR2A receptors with mutations in the pre-M1 region should be completely eliminated by EDTA or other chelators that reduce extracellular free zinc. To our surprise, we observed robust glycine-independent desensitization for this mutant (Fig.1). The ratio of the steady-state current over the peak (Iss/Ipk) for NR2A(AD1) was 0.52±0.04 (n=8) while the time constant was 1.70±0.15 sec (Fig.1B, C). Neither the degree of desensitization nor the time constant were significantly altered ($p>0.05$). Thus, the pre-M1 region is not critical for glycine-independent desensitization as suggested by earlier studies.

Could the ATD of NR2A then be the molecular determinant for glycine-independent desensitization? To examine the role of the ATD of NR2A, we measured glycine-independent desensitization of a deletion mutant of NR2A (NR2AΔATD) in which the whole ATD had been removed (Fig.1). This mutant also exhibited normal glycine-independent desensitization with a I_{ss}/I_{pk} of 0.62±0.05 and a time constant of 2.02±0.20 sec (n=7). Thus, the degree of desensitization and the time constant of NR2AΔATD were similar to those of the wild type NR1/NR2A receptor (Fig.1B, C; $p>0.05$), suggesting that the ATD of NR2A has no direct role in desensitization of NMDA receptors. Taken together, our data suggest that the pre-M1 region and the ATD of NR2A play no significant role in glycine-independent desensitization, and the molecular determinants for glycine-independent desensitization are located elsewhere.

The *lurcher* mutation of either NR1 or NR2A reduces glycine-independent desensitization

It has been reported that the lurcher point mutation of either NR1 or NR2A eliminated the fast component of apparent desensitization (Kohda et al., 2000). However, it remains unclear whether these mutations also alter the slow glycine-independent desensitization of NR1/NR2A

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receptors. In this study, we tested directly whether the single point mutation at the *lurcher* site of either NR1 or NR2A is sufficient to alter glycine-independent desensitization. As shown in Fig.2, the degree of glycine-independent desensitization was significantly reduced by either the *lurcher* mutation of NR1 (NR1a(A653T)) with a I_{ss}/I_{pk} of 0.66±0.2 ($p<0.05$, n=7) or the lurcher mutation of NR2A (NR2A(A651T)) with a I_{ss}/I_{pk} of 0.73±0.04 ($p<0.01$, n=6). The double *lurcher* mutant receptor (NR1a(A653T)/NR2A(A651T)) exhibits greatly reduced glycine-independent desensitization (Fig.2A, B). The I_{ss}/I_{pk} for the double lurcher mutant receptors is 0.89±0.04 ($p<0.001$, n=5). NR1a(A653T) also significantly increases the time constant for glycine independent desensitization (3.42±0.68 sec; $p<0.05$, n=7) while the time constant for glycine-independent desensitization for NR2A(A651T) was not significantly altered (2.12±0.30 sec; $p>0.05$, n=6). Our data suggest that the highly conserved *lurcher* motif is not only critical for the proton-sensitive channel gating as reported previously (Low et al., 2003), but also critical for glycine-independent desensitization of NMDA receptors.

Glycine-independent desensitization and proton-sensitive gating are modified by the same residues in the *lurcher* motif

Our data on the *lurcher* mutants raised the possibility that proton-sensitive gating and desensitization may be closely associated and may share the same molecular determinants. We have previously screened mutants in the lurcher motif of NR1 and NR2A and identified two residues that are critical for proton-sensitive gating of NMDA receptors (i.e. NR1a(T648C) and NR1a(A649C) and corresponding residues in NR2A; Low et al., 2003; Hu & Zheng, 2004). If proton-sensitive gating and desensitization are closely associated, these two residues should also play a critical role in glycine-independent desensitization. To test our hypothesis, we determined

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the effects of these mutations on glycine-independent desensitization under zinc-free conditions. The first pair of mutants tested were NR1a(T648C) and NR2A(T646C). As shown in Fig.3A and B, the degree of desensitization was significantly reduced by these two point mutations ($p<0.001$). The I_{ss}/I_{pk} was 0.75±0.04 for NR1a(T648C) (n=5) and 0.74±0.02 for NR2A(T646C) (n=17). The onset of desensitization for these mutants also appears to be slower than the onset of desensitization for wild type receptors. Preliminary curve fitting suggested that time constants for desensitization were greater than 5 sec. Since glutamate was applied for only 4.5-5 seconds, the decay of outward currents did not reach the steady state at the end of glutamate application, and time constants obtained for these mutants were not reliable. We tried to express the double mutants at this residue but failed to obtain any viable cells due to excessive cell death. NR1a(A649C) and NR2A(A647C) result in even greater reduction in glycine-independent desensitization (Fig.4A,B). The I_{ss}/I_{pk} was 1.00±0.02 for NR1a(A649C) (n=6) and 0.96±0.03 for NR2A(A648C) (n=5). In fact, the desensitization was completely abolished in most cells recorded as shown in the sample current traces in Fig.4A.

To determine whether other residues in the *lurcher* motif also are critical for glycine-independent desensitization, we screened cysteine substitution mutants of NR1aY647, NR1aN650, NR1aL651, NR1aA652, NR1aA653 and NR1aF654. NR1a(Y647C)/NR2A receptors expressed in HEK 293 cells exhibited very small whole cell currents that make the analysis of glycine-independent desensitization unreliable. The effects of the remaining mutants are shown in Fig.5. The degree of desensitization of these mutants was not significantly different from the degree of desensitization of wild type NR1/NR2A receptors (Fig.5A; $p>0.05$). The time constants for glycine-independent desensitization were also not significantly altered by these mutations (Fig.5B; $p>0.05$). Thus, NR1a(T648C) and NR1a(A649C) are the only cysteine

substitution mutants among all tested ones in the *lurcher* motif of NR1 that alter the glycine-independent desensitization of NR1/NR2A receptors.

DISCUSSION

The desensitization of NMDA receptors is complex and involves several distinct processes. For clarity, we defined glycine-independent desensitization as the slow relaxation of the outward macroscopic NMDA receptor current in the continuous presence of glutamate and glycine that can be well fitted with a single exponential component (Zheng et al., 2001). The fast component of desensitization of NR1/NR2A receptors with a time constant of 200-300 ms was described as glycine-independent desensitization in earlier studies but is actually caused by an allosteric interaction between the zinc binding site in the ATD and the glutamate binding site in the S1/S2 domain (Zheng et al, 2001). The goal of this study was to elucidate the molecular determinants of the slow component of desensitization with a time constant of 2 sec. This slow component of desensitization exhibits significant cell-to-cell variability, and marginal changes are difficult to detect. However, we can detect significant changes in glycine-dependent desensitization with high confidence ($p<0.01$).

The first surprising finding of this study is that the two domains in NR2A that have been suggested as the molecular determinants for glycine-independent desensitization are not required. We expected that the mutations in the pre-M1 region would completely abolish the glycine-independent desensitization. However, the NR2A(AD1) had no effect on either the degree or the time constant of glycine-independent desensitization. We cannot completely rule out subtle effects on glycine-independent desensitization by mutations in the pre-M1 region. However, our data strongly suggest that the pre-M1 region is not critical for the glycine-

independent desensitization as suggested by earlier reports (Krupp et al., 1998; Villarroel et al., 1998). Deletion of the ATD of NR2A also had no effect on glycine-independent desensitization. This was not unexpected since there is ample evidence that the ATD is involved in the allosteric interaction between the zinc binding site and the glutamate binding site that results in the fast component of desensitization (Zheng et al., 2001).

Our data suggest that the role of the pre-M1 region in desensitization suggested by those earlier studies needs to be revised. Since the glycine-independent desensitization is not altered, the only component that could be altered by NR2A(AD1) would be the fast component of desensitization that is caused by the allosteric interaction between the zinc binding site in the ATD and the glutamate binding site in the S1/S2 domain of NR2A. Thus, the pre-M1 region and the ATD of NR2A may both be critical for the fast component of desensitization that is caused by ambient zinc acting at the extracellular high affinity site located in the ATD of NR2A. How the mutations in the pre-M1 region reduce the fast component of desensitization remains to be determined. Given the complex nature of the allosteric interaction between the binding sites for zinc, glutamate and proton, there are many possible explanations for the apparent reduction of the fast zinc-dependent desensitization. For example, a reduction in proton sensitivity would result in a reduction in the degree of the fast component of desensitization. Additional studies are needed to elucidate the exact mechanism by which the pre-M1 mutations alter the zinc-dependent apparent desensitization.

Since the pre-M1 region and the ATD of NR2A are not the critical molecular determinants for glycine-independent desensitization, we looked in other regions of the NMDA receptor. The *lurcher* motif located near the extracellular end of the third membrane-spanning region (M3) stands out since it is critical for the gating of glutamate receptors (Kohda et al., 200;

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Taverna et al., 2000; Jones et al;, 2002). A previous study (Kohda et al., 2000) suggested that *lurcher* mutations alter desensitization of NMDA receptors. However, it remained unclear whether the glycine-independent desensitization or the fast zinc-dependent apparent desensitization is reduced by these mutations. Our data clearly suggest that glycine-independent desensitization is reduced by the *lurcher* point mutation in either NR1 or NR2A subunit. This finding prompted us to systematically examine other residues in the lurcher motif. We identified two additional residues in the lurcher motif (NR1aT648, NR1aA649 and corresponding residues in the NR2A subunit) that play a critical role in glycine-independent desensitization. In particular, a single point mutation at NR1A649 or NR2AA647 abolished desensitization. These two mutants had the greatest effect on desensitization of any single point mutations. Taken together, our data suggest that three residues in the highly conserved *lurcher* motif play a critical role in glycine-independent desensitization. Thus, the molecular determinants for glycine-independent desensitization appear to be located in the same regions that are critical for channel gating of NMDA receptors. The role of the *lurcher* motif in desensitization may be a common feature of all glutamate receptors. For instance, a recent study (Klein and Howe, 2004) has shown that the *lurcher* motif is critical for the desensitization of AMPA receptors.

It also is interesting that five of these six mutants share the following common characteristics: (a) slower deactivation time constants (Hu and Zheng, 2004); (b) reduced proton sensitivity (Low et al., 2003); and (c) reduced glycine-independent desensitization. While it is possible that these common characteristics could be just coincidental, we propose that it is an indication for a close link between these processes at a molecular level. Shared molecular determinants for these processes have also been reported outside the *lurcher* motif recently (Chen et al., 2004). A pore mutant (NR1(N598R)) showed reduced desensitization and increased

deactivation time constant (Chen et al., 2004), as well as reduced proton sensitivity (Kashiwagi et al., 1997). It is possible that the reduction of desensitization can be accounted for by a change in the gating rate. Most of the mutations except NR2A(A651T) may reduce the channel closing rate and therefore shift more receptors into the open state. Such a shift would reduce the proportion of receptors in protonated states as well as the proportion of receptors in the desensitized state. Subsequently the degree of desensitization will be reduced. However, this explanation may be overly simplistic. For instance, it would not explain the effect of NR2A(A651T) on desensitization. It also could not explain the changes in the time constant of glycine-independent desensitization caused by NR1a(A653T). We favor an alternative hypothesis that the rate of desensitization and the rate of channel closing are determined by shared structural elements. In our structural model of the pore regions of NMDA receptors, two residues (NR1aT648, NR1aA649) from the *lurcher* motif and a residue (NR1aN598) from the M2 region cluster together to form the deep end of the outer vestibule (Fig.6). This part of the vestibule may undergo significant conformational changes during gating events (Beck et al., 1999; Sobolevsky et al., 2002). We propose that the deep end of this outer vestibule is one of the shared structural elements for proton-sensitive gating and glycine-independent desensitization. The exact nature of the conformational changes that occur at this structural element remains to be elucidated.

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Footnotes:

a. This work was supported by NINDS (NS39418, FZ).

b. Address reprint requests, correspondence, and requests for materials to Dr. Fang Zheng, Dept. of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 West Markham Street, Little Rock, AR 72205-7199, USA.

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Figure Legends

Fig. 1. Normal glycine-independent desensitization for NR2A(AD1) and NR2AΔATD.

A: Representative current responses for wild type NR1/NR2A receptors, NR1a/NR2AAD1 receptors and NR1a/NR2AΔATD receptors are shown. NMDA receptor currents were recorded in HEK 293 cells expressing wild type or mutant receptors (V_h=+40 mV or +50 mV). A rapid perfusion system (Burleigh LSS3100) applied 100 μM glutamate for 4.5 sec. Glycine (30-60 μM) was present all the time. Free zinc in the recording solution was considered to be "0" nM in the presence of 10 μM EDTA or 10 mM tricine.

B: The degree of desensitization is shown as the ratio of the steady state current measured at the end of the glutamate application (4.5-5 sec) over the peak current (p>0.05, ANOVA; n=22, 8, 7 for wild type NR1/NR2A (WT), NR1/NR2A(AD1) (AD1) and NR1/NR2AΔATD (ΔATD), respectively).

C: Time constants were obtained through curve fitting of the outward currents recorded at +40 or +50 mV with a single exponential component. There was no significant difference in the time constant for the wild type NR1/NR2A, NR1/NR2AAD1 and NR1/NR2ADATD (p>0.05, ANOVA).

Fig.2. Effects of the *lurcher* mutation on desensitization of NR1/NR2A receptors.

A: Representative current traces for NR1a/NR2A(A651T) receptors, NR1a(A653T)/NR2A receptors, and NR1a(A653T)/NR2A(A651T) receptors were recorded in HEK 293 cells under nominally zinc-free conditions (V_h=+40 mV, 100 μM glutamate, 4.5 sec). Glycine (30-60 μM) was present all the time.

B: The I_{ss}/I_{pk} of outward currents (V_h=+40 or +50 mV) was determined for wild type NR1/NR2A (WT, n=22), NR1a(A653T)/NR2A (n=7), NR1a/NR2A(A651T) (n=5) and NR1a(A653T)/NR2A(A651T) (n=5). The *lurcher* mutation in either NR1a or NR2A significantly reduced the degree of desensitization (*: p<0.05, **: p<0.01, ANOVA with Tukey *post hoc* test). The desensitization was further reduced by the double *lurcher* mutation (***: p<0.001, ANOVA and *post hoc* test).

C: Time constants for glycine-independent desensitization were determined by curve fitting of the outward currents with a single exponential component. NR1a(A653T) exhibited a significantly greater time constant (*: p<0.05, ANOVA with Tukey *post hoc* test).

Fig. 3. Reduced glycine-independent desensitization of NR1a(T648C)/NR2A and NR1a/NR2A(T646C) receptors.

A: Representative current traces for NR1a(T648C)/NR2A and NR1a/NR2A(T646C) receptors were recorded in HEK 293 cells under nominally zinc-free conditions (V_h=+40 mV, 100 μM glutamate, 4.5 sec). Glycine (30-60 μM) was present all the time.

B: The I_{ss}/I_{pk} of outward currents (V_h=+40 or +50 mV) was determined for wild type NR1/NR2A (WT, n=22), NR1a(T648C)/NR2A (n=5), NR1a/NR2A(T646C) (n=17). The desensitization was significantly reduced by both mutations (***: p<0.01, ANOVA with Tukey *post hoc* test).

Fig. 4. Glycine-independent desensitization was abolished by NR1a(A649C) or NR2A(A647C).

A: Representative current traces for NR1a(A649C)/NR2A and NR1a/NR2A(A647C) receptors were recorded in HEK 293 cells under nominally zinc-free conditions (V_h=+40 mV, 100 μM glutamate, 4.5 sec). Glycine (30-60 μM) was present all the time.

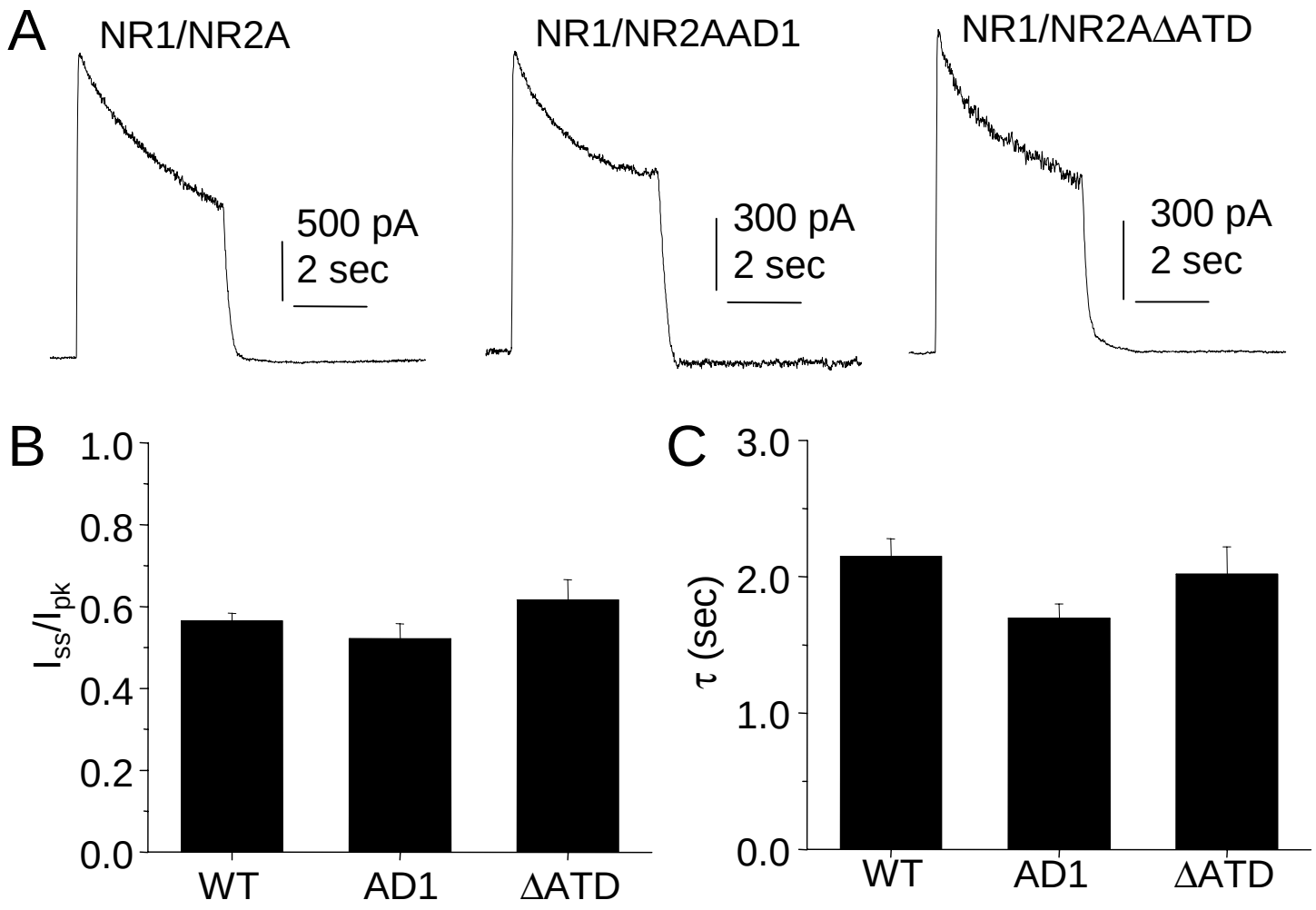
B: The I_{ss}/I_{pk} of outward currents (V_h=+40 or +50 mV) was determined for wild type NR1/NR2A (WT, n=22), NR1a(A649C)/NR2A (n=6), NR1a/NR2A(A647C) (n=5). The desensitization was significantly reduced by both mutations (***: p<0.001, ANOVA with Tukey *post hoc* test).

Fig. 5. Normal glycine-independent desensitization for other cysteine substitution mutants in the *lurcher* motif of NR1.

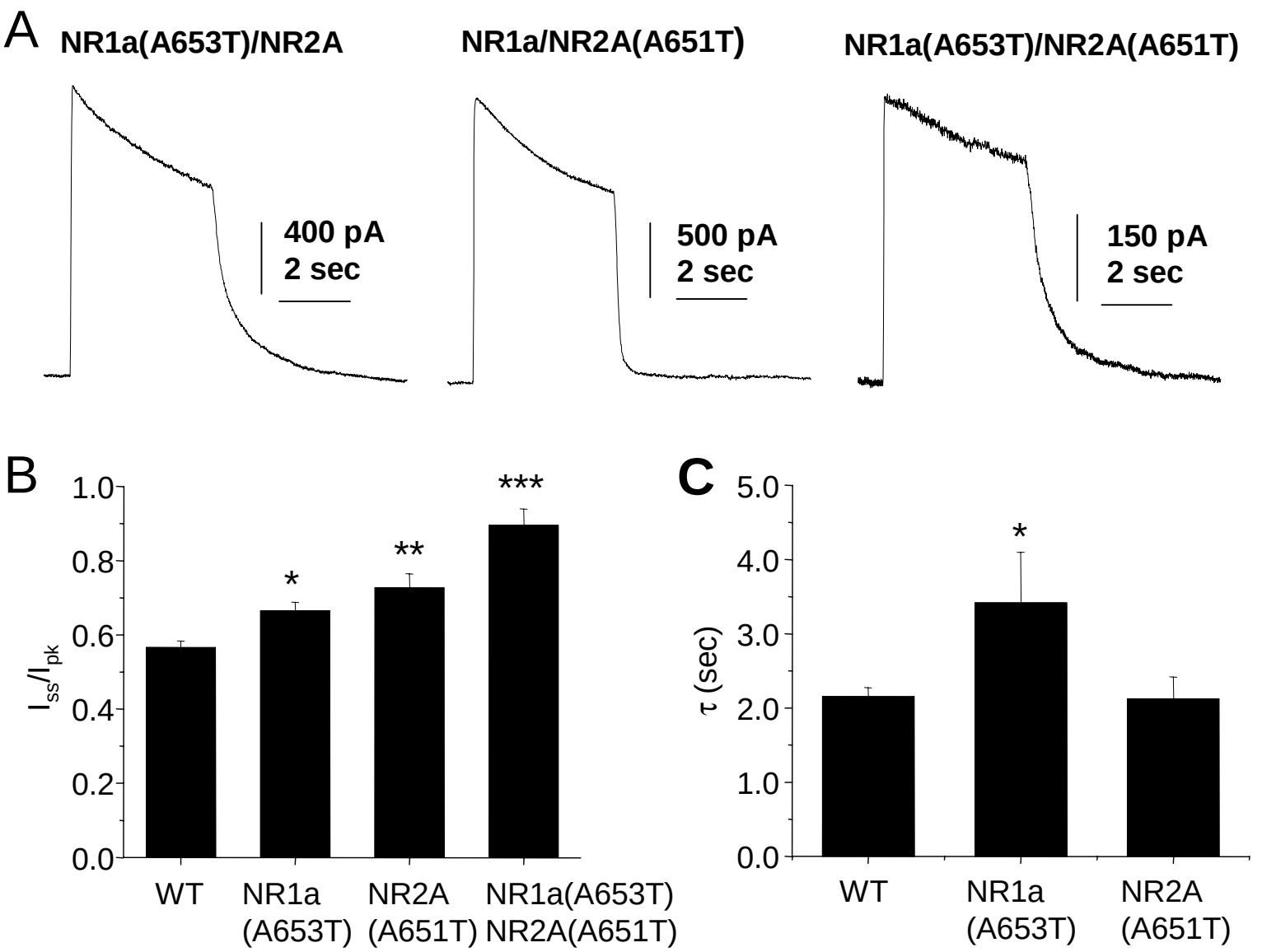
The I_{ss}/I_{pk} (A) and time constants (B) were determined for the outward NMDA receptor currents recorded in HEK 293 cells (V_h=+40 mV or +50 mV, 100 μM glutamate, 4.5 sec). Glycine (30-60 μM) was present all the time. There was no significant change of either the I_{ss}/I_{pk} or the time constants in any of these 5 mutations (p>0.05, ANOVA; n=6, 6, 7, 9, 6 for NR1aN650C, NR1aL651C, NR1aA652C, NR1aA653C, NR1aF654C respectively).

Fig. 6. Homology model for the pore region of NMDA receptors based on the crystal structure of MthK. The M1, M2 and M3 regions from two opposite NR1 subunits are shown as cartoons in "A". The asparagine residue at the Q/R/N site of NR1 (NR1aN598) located at the top of the M2 reentrance loop, NR1aT648 and NR1aA649 are shown as sticks and balls. The *lurcher* motif is colored blue. A side-view space-filled model was shown in "B" in the slab mode across the center of the pore region. NR1aN598, NR1aT648 and NR1aA649 (and

corresponding residues in NR2A) were colored green, cyan and purple respectively. These three residues form the bottom part of the outer vestibule.



JPET80168 Figure1



JPET80168 Figure2

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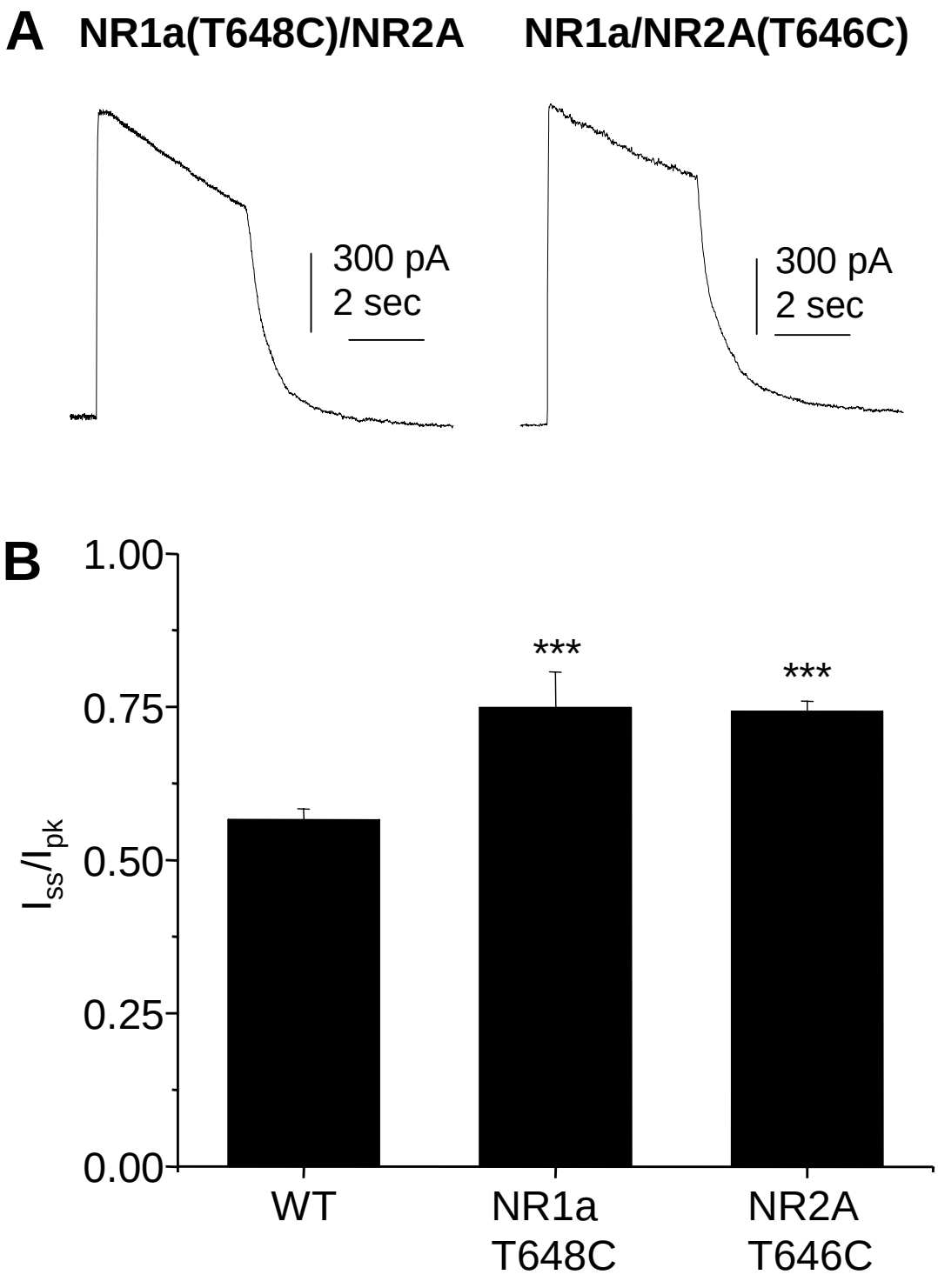

JPET80168 Figure 3

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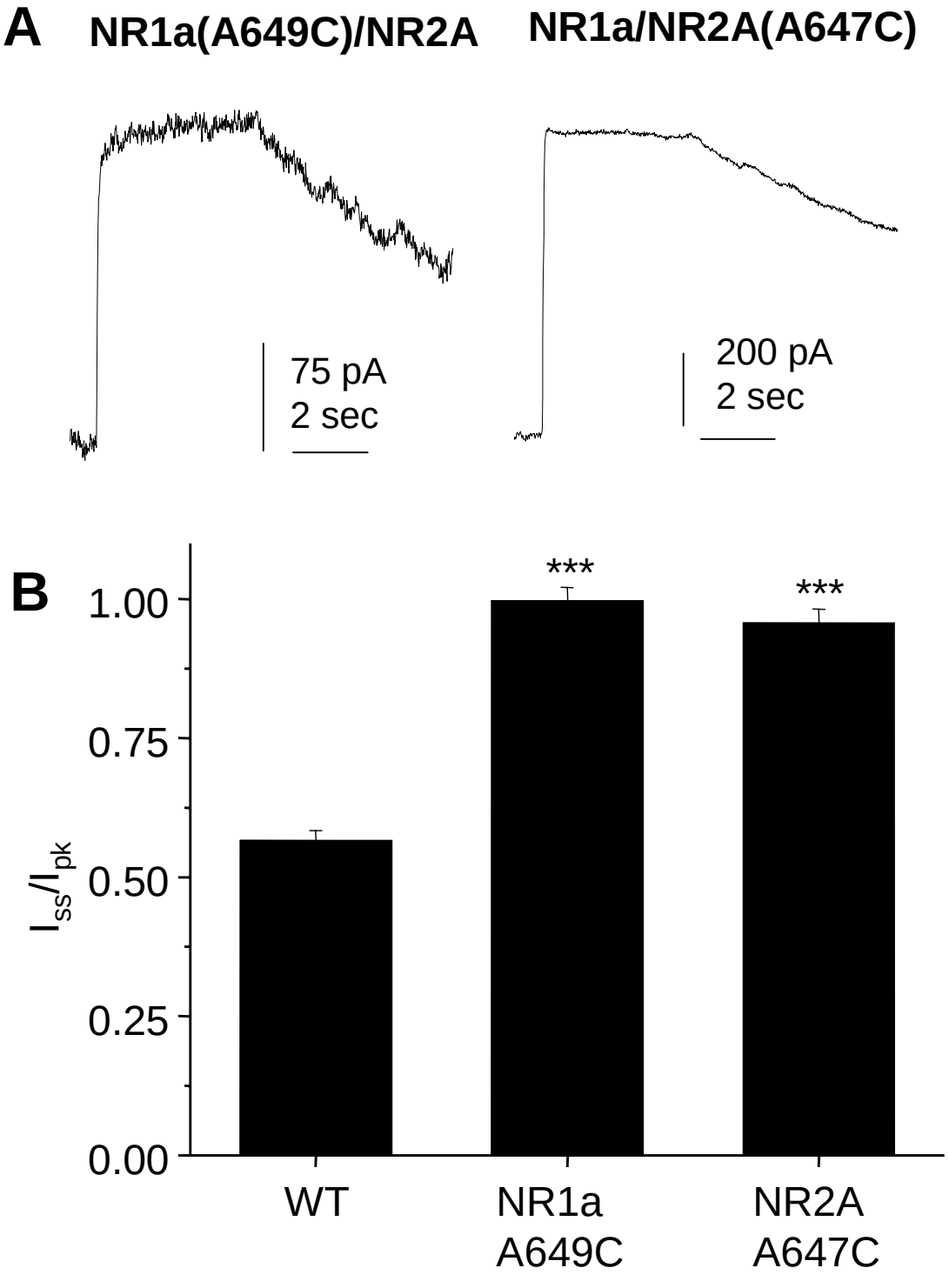

JPET80168 Figure 4

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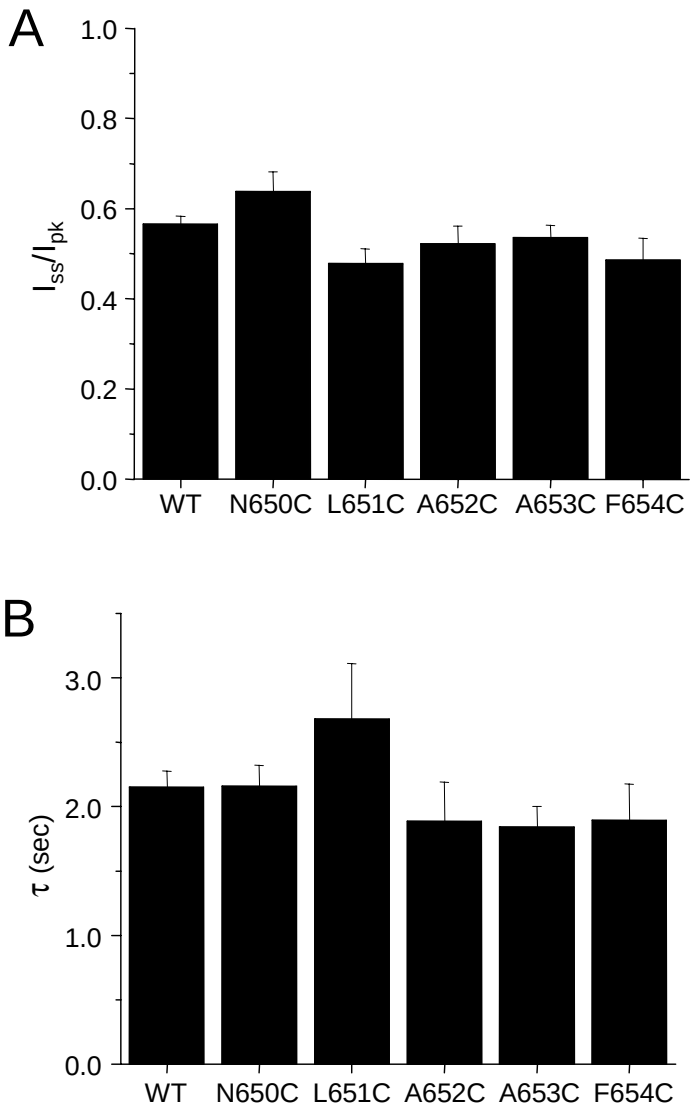

JPET80168 Figure 5

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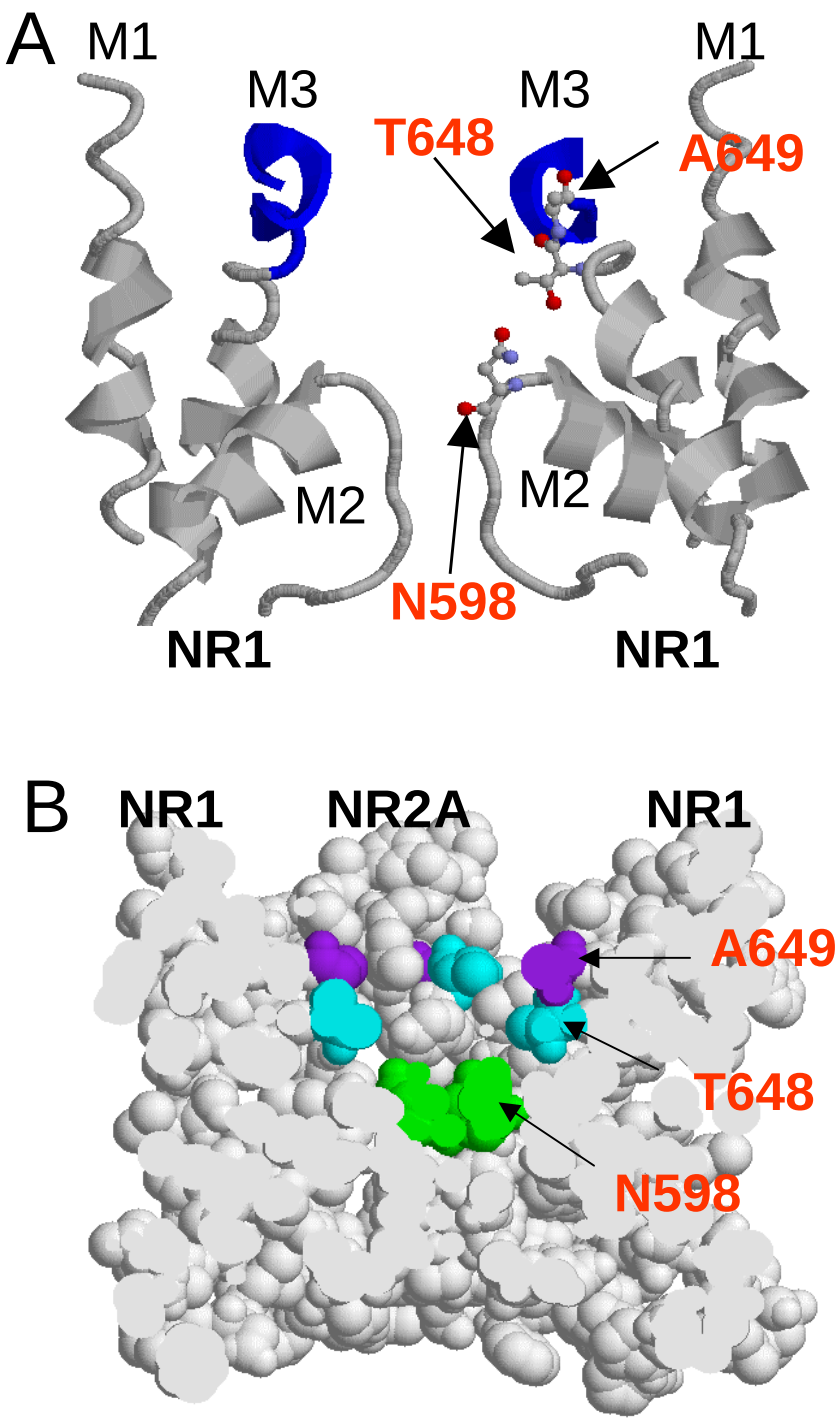

JPET80168 Figure 6