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The selectivity of conantokin-G for ion channel inhibition of NR2B subunit-containing NMDA receptors is regulated by amino acid residues in the S2 region of NR2B

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Abstract

The conantokins are short, naturally-occurring peptides that inhibit ion flow through N-methyl-D-aspartate receptor (NMDAR) channels. One member of this peptide family, conantokin-G (con-G), specifically antagonizes NR2B-containing NMDAR channels, whereas other known conantokins are less selective inhibitors with regard to the nature of the NR2 subunit of the NMDAR complex. In order to define the molecular determinants of NR2B that govern con-G selectivity, we evaluated the ability of con-G to inhibit NMDAR ion channels expressed in human embryonic kidney (HEK)293 cells transfected with NR1, in combination with various NR2A/2B chimeras and point mutants, by electrophysiology using cells voltage-clamped in the whole cell configuration. We found that a variant of the con-G-insensitive subunit, NR2A, in which the 158 residues comprising the S2 peptide segment (E⁶⁵⁷-I⁸¹⁴) were replaced by the corresponding S2 region of NR2B (E⁶⁵⁸-I⁸¹⁵), results in receptors that are highly sensitive to inhibition by con-G. Of the 22 amino acids that are different between the NR2A-S2 and the NR2B-S2 regions, exchange of one of these, M⁷³⁹ of NR2B for the equivalent K⁷³⁸ of NR2A, was sufficient to completely import the inhibitory activity of con-G into NR1b/NR2A-containing NMDARs. Some reinforcement of this effect was found by substitution of a second amino acid, K⁷⁵⁵ of NR2B for Y⁷⁵⁴ of NR2A. The discovery of the molecular determinants of NR2B selectivity with con-G has implications for the design of subunit-selective neurobiological probes and drug therapies, in addition to advancing our understanding of NR2B- versus NR2A-mediated neurological processes.

Keywords

conantokins; NMDAR subtypes; electrophysiology; ion channels; NMDAR mutagenesis

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¹Nonstandard abbreviations: NMDAR, N-methyl-D-aspartate receptor; NR1, N-methyl-D-aspartate receptor subunit 1, with its 8 splice variants (a–h); NR2, N-methyl-D-aspartate receptor subunit 2, with its 4 gene products (A–D); NR3, N-methyl-D-aspartate receptor subunit 3, with its two variants, A and B; NTD, N-terminal domain of NR subunits; S1 and S2 extracellular regions 1 and 2, respectively, of NR subunits; TM, transmembrane domain of NR subunits; Glu, γ-carboxyglutamate; HEK293, human embryonic kidney 293 cells; GFP, green fluorescent protein; WT, wild-type.

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1. Introduction

The NMDAR is a ligand-gated ion channel that belongs to the family of ionotropic glutamate receptors. In the absence of Mg^{2+} , open NMDAR channels show a small degree of voltage-dependent gating, with depolarization enhancing the probability of open channels (Clarke and Johnson, 2008). However, at physiological levels of Mg^{2+} , NMDA/glycine-induced current flow through the ion channels is strongly voltage-dependent, requiring membrane depolarization to relieve the channel block by Mg^{2+} (Mayer et al., 1984). Ca^{2+} influx through NMDAR channels then transduces signaling cascades (Sattler et al., 1999), which, in-turn, establish numerous physiological events, e.g. synaptic plasticity in the brain and spinal cord, with important implications for a variety of processes, such as learning and memory, as well as the perception of pain.

In rat brain, three subunit families of NMDARs, NR1, NR2, and NR3, have been identified. Functional NMDAR ion channels are formed as heterotetramers containing 2 copies of NR1 and 2 copies of NR2 and/or NR3. These subunits are structurally diverse. Within the NR1 family, differential splicing of 4 of the 22 NR1 exons yields 8 different variants of NR1 (NR1a-NR1h), with the largest of these proteins containing 938 amino acids (Hollmann et al., 1993; Sugihara et al., 1992). Four different gene products comprise the NR2 subunit family, and are designated NR2A-NR2D. These range in primary structure from 1218–1456 amino acids, and display 26–27% sequence homology to NR1 (Hollmann and Heinemann, 1994). Two NR3 subunits have been identified, NR3A and NR3B (Matsuda et al., 2003). Because of the complexity of this receptor, and the numerous subunit associations that have been identified, ligand responses rely on specific NR subunit combinations (Furukawa and Gouaux, 2003; Hirai et al., 1996).

The natural subunit composition of the NMDAR in brain shows temporal and spatial dependency in health and disease, and the exact subunit composition of the NMDAR, particularly the presence of NR2B, dictates many of the properties of the receptor. These findings have provided impetus for the development of allosteric NR2B-selective NMDAR antagonists. Because such agents would target only a subpopulation of NMDARs, fewer side effects may prevail in comparison with non-selective NMDAR inhibitors.

Based on homology with other receptors, a structural model of NR1 has been proposed (Wo and Oswald, 1994), that generally applies to all NR1 and NR2 subunits, which includes an 18-residue signal peptide, followed by a N-terminal extracellular portion (NTD/S1; 543-residues) (Kuryatov et al., 1994). Residues 19–375 (NTD), contain regulatory domains (R1 and R2) (Masuko et al., 1999), and residues 376–543 comprise the extracellular S1 region. Immediately downstream of the NTD/S1 region, a TM domain (TM1; 19-residues) is present, followed by a 19-residue linker and a second hairpin-like TM domain (TM2; 21-residues) that neither spans the membrane nor reenters the extracellular space (Wood et al., 1995). Next, a 10-residue intracellular linker precedes a third TM domain (TM3; 19-residues) that spans the membrane. This allows projection of a TM linker (S2; 163-residues) into the extracellular region (Wood et al., 1997). Finally, a fourth TM domain (TM4; 21-residues) precedes a C-terminal intracellular segment (105-residues) that contains phosphorylation sites (Tingley et al., 1997).

The venoms of the predatory marine cone snails (genus *Conus*) contain numerous structurally and pharmacologically diverse peptides, the conopeptides, that target specific ligand- or voltage-operated ion channel receptors (reviewed in: (Prorok and Castellino, 2007; Terlau and Olivera, 2004). One family of these peptides, the conantokins, are disulfide-poor, rich in Glu, and specifically antagonize the NMDAR. The known members of the conantokin (con) family are: con-E, con-G, con-R, con-L, con-P, con-Pr1, con-Pr2, con-Pr3, and con-T (Gowd et al.,

2008; Haack et al., 1990; Jimenez et al., 2002; McIntosh et al., 1984; Teichert et al., 2007; White et al., 2000), with varying patterns of NR2 subtype selectivity. The conantokins have shown some clinical potential, particularly con-G, e.g., for the treatment of pain and convulsions after seizure, and for protection against apoptosis of neurons after ischemic stroke (Barton et al., 2004; Malmberg et al., 2003; Williams et al., 2002). This is likely due to the apparent NR2B subunit selectivity of con-G, a property that may contribute to its greater therapeutic index in the mouse models compared with the less selective con-T (Donevan and McCabe, 2000; Klein et al., 2001; Sheng et al., 2007).

Despite the important implications inherent in the development of NR2B-selective therapeutic agents, the molecular basis of conantokin subunit selectivity is not well understood. Thus, the current investigation was designed to uncover the features of NR2B that govern its high selectivity for con-G by attempting to engineer these features into a less selective NMDAR subunit, NR2A. This manuscript provides a summary of the results obtained.

2. Materials and methods

2.1 Peptide synthesis

The amino acid sequences of the parent conantokins that were chemically synthesized for these studies are provided below (Gla = γ).

Solid phase peptide synthesis was employed using an Applied Biosystems (Foster City, CA) model 433A peptide synthesizer. All reagents and methods have been described previously (Prorok et al., 1996).

2.2 Plasmid constructs

cDNAs encoding rat NR1a, NR1b, NR2A, and NR2B were provided by Dr. David Lynch (University of Pennsylvania). Routine molecular biology methods were used to construct chimeras of NR2A and NR2B, via replacement of residues 657–814 (S2) in the NR2A subunit with residues 658–815 (S2) of the NR2B subunit (referred to as NR2A[2B-S2]) and, conversely, by replacement of the NR2B-S2 region with the equivalent sequence from the NR2A subunit (NR2B[2A-S2]). A similar set of chimeras was produced that contained an NR2A-S1 replacement in the NR2B background and an NR2B-S1 replacement in the NR2A background (NR2B[2A-S1] and NR2A[2B-S1], respectively). In addition, two additional chimeric constructs were generated in which the first and second halves of NR2B-S2 regions [residues 658–727 (NR2B-S2(a)) and residues 728–815 (NR2B-S2(b)), respectively] were exchanged with those of the NR2A subunit [residues 657–726 (NR2A-S2(a)) and residues 727–814 (NR2A-S2(b)), respectively]. These two chimeras are referred to as NR2A[2B-S2(a)] and NR2A[2B-S2(b)], respectively. The protein constructs are diagrammed according to their domain components in Fig. 1.

Point-mutations in the plasmids for NR2A and NR2B were created by site-directed mutagenesis. All final sequences were verified by DNA sequence analysis.

2.3 Expression of recombinant (r) NMDAR subtype

Plasmid pEGFP-N1, encoding a red-shifted variant of wild-type (WT) green fluorescent protein (GFP), was purchased from Clontech Laboratories (San Diego, CA). HEK293 cells

were transiently transfected with recombinant NR subunits using calcium phosphate precipitation (Sheng et al., 2007). The cells were grown to ~50% confluency on 60 mm poly-D-lysine-coated dishes and transfected with combinations of NR1a, NR1b, NR2A, NR2B, and chimeric NR2 subunits that contained plasmids (10 µg of DNA/dish), along with pEGFP-N1 for positive selection of transfected cells. Plasmid ratios for diheteromeric combinations were 1:3:6 for GFP:NR1a/b:NR2A/B (and chimeras of NR2 subunits), respectively. Following transfection, the cells were maintained in fresh medium containing 500 µM ketamine to prevent the glutamate that was present in the culture medium from stimulating cell death. After 48 hr post-transfection, the cells in 60 mm dishes were plated in 35 mm poly-D-lysine-coated dishes for electrophysiological recordings.

2.4 Electrophysiology

Electrophysiological patch clamp whole cell recordings were performed at room temperature using equipment previously described (Sheng et al., 2007). The extracellular solution for the cells consisted of 140 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 10 mM Na-Hepes, and 20 mM dextrose, pH 7.35. In some cases, 100 µM MgCl₂ was added to this solution. Glycine (10 µM) was also present to saturate its site on the NMDAR. Since we obtain an *EC*₅₀ for glycine of 1 µM, the level of 10 µM used in these experiments is approaches saturation. Additionally, since glycine is not competitive with con-G or con-T, the concentration used in all experiments is suitable for the purposes intended. Borosilicate recording pipettes, with a resistance of 2–4 ΩM, were back-filled with a solution containing 140 mM CsF, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, 10 mM Cs-Hepes, 2 mM tetraethylammonium chloride, and 4 mM Na₂ATP, pH 7.35. Stock solutions of 10 mM NMDA and 10 mM glycine prepared daily.

Agonist solutions were applied at flow rates of 2.5 ml/min using a rapid solution changer equipped with a 9-barrel straight-head (RSC-200, Molecular Kinetics, Pullman, WA) positioned 200–300 µm from the cell under study. Whole-cell currents were recorded using an Axopatch-200B amplifier (Axon CNS/Molecular Devices, Sunnyvale, CA), low-pass filtered at 5 kHz by a built-in, eight-pole Bessel filter, digitized at a 0.5–2 kHz sampling frequency using a Digidata 1322A (Axon). The data were acquired on a personal computer using pCLAMP 8 software (Axon). Experiments were performed with cells clamped at –70 mV, pH 7.35, 25°C, or as specified. The responses were normalized to the maximum current of a given cell evoked in the absence of peptide. The time resolution of our perfusion system is 4 ± 1 ms (10–90% solution exchange time), which assures that solution exchange equilibrium is reached immediately, relative to the slower on- or off-rates of inhibitors with time constants of the magnitude of seconds.

Generally, we employed a model with a single functional receptor site for the conantokins in order to calculate the onset and offset time constants for conantokin inhibition of NMDA-evoked currents (Sheng et al., 2007). For those cases with τ_{off} values >30 sec, we measured onset-of-inhibition times (τ_{obs}) at several conantokin concentrations and employed the equation:

$$1/\tau_{\text{obs}} = k_{\text{on}}[\text{conantokin}] + k_{\text{off}}$$

to obtain kinetic association and dissociation inhibition rate constants of conantokins with various recombinant NMDARs. A plot of $1/\tau_{\text{obs}}$ versus the concentration of conantokin allowed k_{on} and k_{off} values for conantokin-inhibition of NMDA/glycine-stimulated ion channel currents to be calculated from the slopes and intercepts, respectively (Sheng et al., 2007). Values for K_i were derived from $k_{\text{off}}/k_{\text{on}}$.

2.5 Statistics

Statistical analyses were performed using the two-tailed Student's t-test, and repetitive measures of analysis of variance (Sigma Stat, Jandel Scientific, San Rafael, CA; and Origin software). Significance was assigned at $P < 0.05$.

3. Results

Previous studies have shown that con-T is nonselective for inhibition of ion channels in NMDARs composed of NR1/NR2A or NR1/NR2B, but con-G is selective for NR1/NR2B-containing subunits (Sheng et al., 2007). As seen in Figure 2, inhibition by con-G of NMDA/glycine-induced current flow through recombinant NMDARs, reconstituted from specific subunit combinations by transfection in HEK293 cells, only occurs in receptors containing NR2B subunits, regardless of whether the NR1 component is NR1a or NR1b. The competitive nature of the inhibition by con-G with respect to glutamate/NMDA has been previously established in NR2B-containing receptors (Donevan and McCabe, 2000). These data establish the foundation for the experiments that follow that are focused on assessment of the necessary features of the NR2B subunit that serve as determinants of its specificity toward con-G.

To assess the functional determinants of the NR2B subunits for con-G specificity, a reductionist approach was followed, where peptide segments were exchanged between the con-G-insensitive NR2A and the con-G-sensitive NR2B subunits, followed by examination of the electrophysiological response of the chimeric NMDARs toward con-G. For most experiments, constructs were generated that only contained mutations in the S1 and S2 domains of the NR2 subunits, since these regions of the protein, along with the NTD, are the only extracellular domains of NR2, and thus likely contain the binding determinants of soluble regulatory proteins, peptides, and small molecules. Since this work focused primarily on the S2 region of NR2, the amino acid sequences of S2 of NR2A and NR2B are provided in Fig. 3A, with the amino acid differences between the segments indicated in red lettering. S2(a) and S2(b) represent two halves of the S2 sequence that were treated separately.

The data obtained for con-G inhibition of the NMDA/glycine-stimulated whole cell ion channel currents that resulted from replacing various segments of NR2A with those of NR2B, in combination with NR1b, are illustrated in Fig. 3B–E. Replacement of the entire S1 segment of NR2A with that of NR2B provided electrophysiology profiles (Fig. 3B) for con-G inhibition that were similar to the null response of NR1b/NR2A to the application of 20 μ M con-G (Fig. 2C). Thus, we conclude that the S1 region of NR2B does not contain determinants for the inhibitory effects of con-G. However, generation of a chimeric NR2A that contained S2 of NR2B, i.e., NR2A[2B-S2], in combination with NR1b, showed virtually identical ion current inhibition by 20 μ M con-G (Fig. 3C) as the NR1b/NR2B combination in transfected HEK293 cells (Fig. 2D). Further, the data of Fig. 3D clearly demonstrate that con-G sensitivity is incorporated into NR2A when the S2(b) region of NR2B replaced the S2(b) region of NR2A, in otherwise intact NR2A. Conversely, replacement of NR2A-S2(a) with NR2B-S2(a) in NR2A (Fig. 3E) did not confer con-G sensitivity in the chimeric receptor subunit. Thus, the determinants for con-G sensitivity reside in the S2(b) segment of NR2B.

The next step in this approach was to replace more limited sections of NR2A-S2(b) with equivalent regions of NR2B-S2(b). A list of the amino acid substitutions from NR2B into NR2A are provided in Table 1, and comprise all of the differences in the S2(b) regions between NR2A and NR2B. In each case, the onset rates and offset times for con-G inhibition of current flow in HEK293 cells transfected with NMDARs consisting of NR1b and NR2 variants were determined, and the K_i values for con-G were calculated as the ratios of these parameters. Of all the substitutions made, the minimal alteration required to incorporate maximal con-G sensitivity into NR2A is a single amino acid change of K738 of NR2A to Met (Fig. 4A), which

exists at the equivalent sequence position (residue 739) of NR2B. An additional smaller level of con-G sensitivity is incorporated into NR2A via a change of Y754 of NR2A to the equivalent K755 of NR2B (Fig. 4B), and the combination of both amino acid changes are also functional in that regard (Fig. 4C). However, the K738M mutation in NR2A is sufficient in and of itself for con-G sensitivity to be incorporated into NR2A.

Guided by the above results, mutations were made in NR2B with corresponding relevant residues of NR2A to determine if a correlative loss in con-G sensitivity by NR2B would result. As shown in Fig. 4D and Table 1, replacement of NR2B-S2 with NR2A-S2 alters the profile of the con-G response of the chimeric receptor as compared with the parent NR1b/NR2B. While con-G retains its inhibitory effect, the receptor recovery from block, as measured through enhanced current flow after washout of con-G, becomes very rapid, thus increasing the K_i value for con-G inhibition of ion flow through open NMDAR channels (Table 1). This implies that NR2A contains determinants in its S2 region that dictate this faster offset for con-G. One important determinant of this effect is K738 of NR2A, since replacement of M739 of NR2B with the equivalent K738 of NR2A shows a much more rapid offset than NR2B-containing receptor, even more than NR2B[2A-S2] (Fig. 4E). On the other hand, substitution of K755 of NR2B with the equivalent Y754 of NR2A has little effect on the inhibitory properties of con-G (Fig. 4F). The receptor containing the double mutant of NR2B, NR2B[M739K/K755Y], is similar to the single point mutation, NR2B[M739K], leading to an 80-fold higher K_i for con-G inhibition (Fig. 4G and Table 1).

We also examined the responses of con-T to these same mutations of NR2B and determined that they are not the same as those of con-G. The data of Fig. 5 show that the replacement of the S2 segment of NR2B with the corresponding segment of NR2A (Fig. 5A,B) led to a dramatic increase in the K_i value for con-G inhibition (from $0.8 \pm 0.4 \mu\text{M}$ for NR1b/NR2B ion channels to $57.6 \pm 7.2 \mu\text{M}$ for NR1b/NR2B[2A-S2] channels; Table 1 and Fig. 5E), primarily due to the faster recovery rate of the NR1b/NR2B[2A-S2] receptors (Fig. 5B). On the other hand, the K_i for con-T ion channel inhibition was essentially unchanged in NMDARs consisting of subunit combinations of NR1b/NR2B ($1.0 \pm 0.4 \mu\text{M}$) and NR1b/NR2B[2A-S2] ($1.8 \pm 0.4 \mu\text{M}$; Fig. 5C–E), as well as NR1b/NR2A ($2.3 \pm 0.7 \mu\text{M}$), and NR1b/NR2A[2B-S2] ($1.6 \pm 0.5 \mu\text{M}$) (Fig. 5E). Data similar to those of con-T were also noted for con-R inhibition of these same NMDAR subunit combinations (not shown). This suggests that the S2 segment of NR2B houses specificity determinants that are unique for con-G.

We probed further into this issue by determining the effects of the aforementioned NR2A and NR2B mutations on the NMDA response properties of the resultant receptor complexes. The EC_{50} values for NMDA at $10 \mu\text{M}$ glycine were obtained from dose-response curves similar to that of Fig. 6A. Interchange of the S1 regions of NR2A and NR2B did not significantly affect the EC_{50} for NMDA. For the NR1b/NR2A receptors, the EC_{50} -NMDA of $23 \mu\text{M}$ was essentially unchanged in NR1b/NR2A[2B-S1] receptors, which displayed an EC_{50} -NMDA value of $26.5 \pm 4.8 \mu\text{M}$. In the case of NR1b/NR2B, the EC_{50} -NMDA of $21 \mu\text{M}$ was elevated to 47.6 ± 5.0 for NR1b/NR2B[2A-S1] receptors. Similarly, it is seen from Fig. 6B that the EC_{50} -NMDA values for the NR1b/NR2A-S2 variants are similar to the EC_{50} value for NMDA toward NR1b/NR2A. However, EC_{50} values for NR1b/NR2B-S2 variants are higher for NMDA than that of the parent NR1b/NR2B. These data suggest that the indicated changes in the S2 region of NR2B alter the response of the receptors for the agonist, NMDA, in NMDARs consisting of these NR2B variants, unlike those observed for similar changes in the S2 of NR2A. Thus, it appears as though exchange of NR2A-S2 residues with corresponding residues of NR2B-S2 does not alter the binding site for NMDA, but substitution of the NR2A-S2 residues for those corresponding to NR2B-S2 reduces the NMDA potency. This effect appears to rely primarily on residues M739 and K755, as a similar diminution of NMDA potency was observed upon replacement of these two residues with their NR2A counterparts. These changes

in the primary structure of NR2B affect both NMDA agonism and con-G inhibition, whereas the complementary alterations in NR2A affect the response to con-G, but not to NMDA.

Similar studies were conducted with the inhibitor ifenprodil, which interacts with the NTD of NR2B subunits (Perin-Dureau et al., 2002), in order to assess whether the mutations in the S1 and S2 domains of NR2 had global effects on inhibitor binding to a distal region of NR2. The 88% inhibition afforded by 10 μ M ifenprodil toward NR1b/NR2B-containing NMDARs (Fig. 7A,E) was virtually identical to that of NR1b/NR2B[2A-S2]-receptors (85%) (Fig. 7B,E), thus showing that substitution of the S2 region of NR2B with that of NR2A did not have long-range effects on the properties of the NTD of NR2B. Whereas, as expected, this same concentration of ifenprodil displayed much less inhibition of NR1b/NR2A NMDARs (not shown), increasing the concentration of ifenprodil to 120 μ M led to approximately 56% inhibition of NR1b/NR2A receptors (Fig. 7C,E). This level of inhibition at the higher ifenprodil level was not affected by substitution of the S2 region of NR2B into NR2A in the NR1b/NR2A[2B-S2] receptor (58%) (Fig. 7D,E), thus confirming the similar lack of long-range effects by the S2 substitutions on the NTD of NR2A.

Lastly, as evidenced by the off-rate of con-G in NR2B variants in which con-G sensitivity was diminished compared to NR1b/NR2B (Table 1), we assessed whether the essential point mutations affected the ion channel directly. This possibility was tested by examining the integrity of the Mg^{2+} channel block with the mutant NMDARs. For this set of experiments, the efficacy of the Mg^{2+} ion channel block was assessed by measuring NMDA/glycine-stimulated current flow in the presence of 100 μ M Mg^{2+} at various holding potentials of the cells. As expected (Nowak et al., 1984), the data of Fig. 8 illustrate that current flow in wild-type NR1b/NR2A (Fig. 8A) and NR1b/NR2B (Fig. 8B) receptors in the absence of Mg^{2+} varies linearly with the holding potential of the cells. Very similar behavior was noted with NR1b/NR2B[M739K] and NR1b/NR2A[K738M] NMDARs. However, in the presence of Mg^{2+} , inward current flow was inhibited at high negative potentials where Mg^{2+} inserts in, and consequently, blocks channel. At positive potentials, the outward current was identical to that obtained in the absence of Mg^{2+} , demonstrating that Mg^{2+} does not block channels in positively polarized cells. These data for the WT NR1b/NR2A and NR1b/NR2B NMDARs were very similar to data from NMDARs containing NR1b, in combination with NR2A[K738M] (Fig. 9C), and NR2B[M739K] (Fig. 8D). Thus, the two latter mutations likely do not affect the ion channel properties of the mutant receptors.

4. Discussion

This study was designed to reveal elements within the NR2 subunit of recombinant NMDARs that dictate the specificity of ion channel inhibition by conantokins. We began with the knowledge that con-T and con-G exhibit different inhibitory characteristics for NMDAR ion channels, with con-G strongly preferring the NR2B subunit for maximal inhibition of current flow, and con-T showing no preference for NR2A or NR2B as the subunit in combination with NR1. Thus, our approach was to search for amino acid residues in the S1 and S2 extracellular domains that differed between the highly homologous NR2B and NR2A, and to use this information to construct NR2B/NR2A chimeras to assess upregulation of con-G selectivity. In terms of their function, regions of the S1 and S2 domains form two lobes of a clamshell in NR subunits and their conformational closing and opening in response to agonists and antagonists are believed to be transmitted allosterically to the opening and closing of ion channels of the NMDAR (Armstrong and Gouaux, 2000).

Initially, the homologous subunits of NR2A and NR2B were aligned and amino acids from NR2B placed into NR2A to assess those changes that generated con-G inhibitory selectivity into NR1/NR2 ion channels. The data of Table 1 show that these determinants of con-G

specificity, at least in major part, are located in the C-terminal region of the S2 segment of NR2B, NR2B-S2(b), between amino acid residues 727–814. At receptors containing NR1b/NR2A[2B-S2(b)] subunit combinations, con-G displayed kinetic constants and maximal steady state levels of NMDAR inhibition that were virtually identical to those of receptors containing the intact NR1b/NR2B subunit combination.

Once this paradigm was established, groups of amino acids in the S2(b) segment from NR2B were substituted into homologous positions in NR2A and the inhibitory properties of con-G examined. The data of Table 1 show that high con-G inhibitory selectivity was incorporated into NR2A by a simple amino acid substitution of the Met that exists at position 739 of NR2B for the equivalent Lys in NR2A. Some reinforcement of con-G selectivity was provided by a second substitution of Lys at position 755 of NR2B for the equivalent Tyr in NR2A, but the M738 replacement in NR2A is sufficient in and of itself to transport con-G selectivity in NR2A. Thus, this single amino acid substitution in NR2A confers con-G ion channel inhibitory selectivity into the NMDAR consisting of NR1b/NR2A, but this does not rule out the possibility that alteration of other residues in the S2 region of NR2A, even those that share identity with NR2B, could also transform the selectivity profile. These same two amino acids (K738 and Y754 in NR2A), when altered to the corresponding amino acids of NR2D, enhanced the potency of glycine as a NR2A variant coagonist by an order of magnitude, approximately the same as that measured for NR1/NR2D receptors (Chen et al., 2008). Thus, K738 and Y754 of NR2A are sites that profoundly alter the specificity properties of both the NR2 and NR1 subunits toward effectors of NMDAR ion channel activity. Both of these amino acids are far removed from the glutamate/NMDA site (Fig. 9) and have not been implicated in the agonist binding pocket of NR2A, the latter of which is most affected by amino acids within regions encompassing amino acid residues 387–493 and 660–709 (Laube et al., 1997), with E387, K459, and K462 also affecting the apparent K_i for con-G inhibition (Wittekindt et al., 2001). Thus, these residues may indirectly affect binding of agonists and antagonists by disrupting the orientation of the ligand binding sites upon opening (inactivating) and closing (activating) the conformation.

As can be further gleaned from the crystal structure of the ligand-binding core of NR2A (Furukawa et al., 2005), K738 and Y754 are separated by a distance of 18–20 Å and, therefore, do not directly interact. These residues are also 12–13 Å from the glutamate/NMDA site, and direct interactions of these residues with the agonist site are also not favored. However, K738 is positioned sufficiently close (2.5–3 Å) to E714 to allow formation of a salt-bridge that, in turn, may constrain NR2A in a conformation that is unfavorable to inhibition by con-G. This electrostatic contact does not exist in NR2B, wherein the subject residues are M739 and D715. Additionally, Y754 of NR2A lies within 4 Å of three other hydrophobic amino acids that are conserved in both NR2 subunits, namely L665, Y698 and M701. This hydrophobic patch of residues may also contribute to the conformation of NR2A and would likely be disrupted in NR2B with the presence of Lys in the position occupied by Y754 in NR2A. A reliable assessment of the conformational distinctions that exist between the NR2 ligand binding cores awaits structural analysis of the NR2B subunit, but it seems likely that the amino acids at positions 738/739 and 754/755 contribute to some degree to the structures of the ligand binding domains of their respective subunits. Furthermore, despite the large distances that separate these sites, the K_i values for con-G (Table 1), that attend the doubly and two singly mutated subunits, suggest that sites 738/739 and 754/755 are not independent. For example, the free energy change accompanying the inhibition constant for con-G as a result of the K755Y replacement in wild-type NR2B ($\Delta G_{wt \rightarrow K755Y} = RT \ln[Ki_{K755Y}/Ki_{wt}]$) is 0.5 kcal/mol, whereas this same replacement in the M739K background is associated with a more favorable free energy ($\Delta G_{M739K \rightarrow M739K/K755Y} = -0.1$ kcal/mol). Likewise, the free energy changes resulting from the M739K replacement in wild-type NR2B and the K755Y mutant are not equivalent ($\Delta G_{wt \rightarrow M739K} = 2.7$ kcal/mol; $\Delta G_{K755Y \rightarrow M739K/K755Y} = 2.1$ kcal/mol). From these

values, a coupling energy of -0.6 kcal/mol is assigned to M739 and K755. Because the distance between these two residues precludes a direct interaction, this coupling energy must ultimately derive from interactions of neighboring residues.

This topic of con-G specificity toward NR2B-containing receptors was further illuminated after examination of whether reverse correlations were at-play with these same amino acid residues, by assessing whether con-G selectivity was attenuated in NR1b/NR2B receptors as a consequence of replacing these same selectivity-inducing residues in NR2B with those that exist in NR2A. The data of Table 1 show that the replacement of M739 of NR2B with K738 of NR2A, greatly increases the K_i value for con-G inhibition of ion channel currents by approximately 80-fold, and results in a decrease in maximal steady state inhibition by con-G to 33% of that for non-mutated NR2B. This substantial increase in the K_i was primarily due to the much more rapid recovery of the ion channels after con-G wash-out, whereas the inhibition on-rate of con-G was essentially the same for NR1b/NR2B and NR1b/NR2B [M739K]. These results can be explained by suggesting that K739 increases the stability of the closed conformation.

We show that the potent inhibition of NR2B-containing NMDAR ion channels by con-G can be adopted by NR2A-containing channels by altering only one amino acid residue in NR2A, K738 to M. However, in NR2B, con-G specificity cannot be completely eliminated by the opposite mutation, M739K, or even with a replacement of the entire S2 region of NR2B by NR2A. Thus, whereas we have identified one critical determinant of the con-G specificity of NR2B, other residues in the S1 region, or even in the N-terminal domain, may be important in themselves for this effect, and/or may be necessary for collaboration with M739 (or other S2 residues) for the NR2B selectivity of con-G. For instance, an amino acid(s) external to S2 may induce subtle conformational effects within the S2 domain that render it more favorable to the toxin. On the other hand, such external elements do not appear to exist in the NR2A subunit, since replacement of the S2 region of NR2A with that of NR2B is sufficient to confer full NR2B-like sensitivity to con-G within the NR2A background. It also seems clear that con-T does not share identical binding site(s) with con-G in NMDARs, since inhibition by con-T is not affected even with both of these residues mutated in NR2A and/or NR2B. This indicates that the diversity of conantokin functioning with the NMDAR is related to both specific sequences of the receptor subunits and of the conantokins.

Finally, we demonstrate herein that the changes introduced in S2 of NR2A and NR2B do not directly affect ion channel permeability, the voltage dependency of the Mg^{2+} block, or distal effects of ligands, e.g., ifenprodil, directed to the NTD of the recombinant NMDARs. However, changes of key residues in NR2B, particularly at M739 and K755, did affect the response of the variant ion channels to NMDA, whereas similar changes in NR2A, at positions 738 and 754, did not result in alterations of the NMDA response to the recombinant variant receptors. Lacking the structure of NR2B, this result cannot be fully explained, but the result is in concert with similar data from a study of NMDA responses in receptors containing NR2A and NR2D (Erreger et al., 2007). In this latter case, it was concluded that the agonist, glutamate, exhibited different binding orientations in these subunits and was differentially affected by ligands and by mutations in the protein subunits. This also may be the case for NR2A and NR2B.

In conclusion, in order to understand the ligand interactions that lead to sensitization and desensitization of NMDAR ion channels, and the specific effectors that lead to receptor subtype selectivity, we took advantage of the high selectivity of con-G for NR2B-containing receptors, and the relatively insensitivity of this same peptide for NR2A-containing receptors. By generating chimeric constructs of NR2A and NR2B, we determined a single amino acid residue from NR2B, M739, accounts for con-G selectivity in NR1b/NR2B-containing receptors and, when inserted into NR2A, confers con-G selectivity to NR1b/NR2A receptors. Further, both

NR2C and NR2D contain M739, and both of these receptor subunits, when combined with NR1, show sensitivity to con-G inhibition (Teichert et al., 2007), thereby confirming the importance of this residue in that regard. Thus, this residue selectively influences channel gating in response to conantokin-type inhibitors, particularly the recovery rate after wash-out of the inhibitor. Coupled with the role of this residue in influencing glycine potency, this region of NR2 appears to be critical for its allosteric functions, and is a potential target for selective actions of pharmacologic NMDAR agents.

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NR2A

NTD	S1	M1	M2	M3	S2	M4	CTD
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NR2A[2B-S1]

NTD	S1	M1	M2	M3	S2	M4	CTD
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NR2A[2B-S2]

NTD	S1	M1	M2	M3	S2	M4	CTD
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NR2A[2B-S2(a)]

NTD	S1	M1	M2	M3	S2(a)	S2(b)	M4	CTD
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NR2A[2B-S2(b)]

NTD	S1	M1	M2	M3	S2(a)	S2(b)	M4	CTD
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NR2B

NTD	S1	M1	M2	M3	S2	M4	CTD
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NR2B[2A-S1]

NTD	S1	M1	M2	M3	S2	M4	CTD
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NR2B[2A-S2]

NTD	S1	M1	M2	M3	S2	M4	CTD
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Fig. 1.

Domain designations of NR2A and NR2B constructs used in this study. NR2A regions are in yellow and NR2B segments are in blue. NTD, N-terminal domain; S1, extracellular domain-1; M1–M4, transmembrane domains 1–4, respectively; S2(a) and S2(b), two peptide segments of extracellular domain-2; CTD, C-terminal domain.

Fig. 2. Inhibition of NMDA (100 μ M)/glycine (10 μ M)-induced ion flow through recombinant NMDAR channels by con-G (20 μ M). The recombinant NMDAR consisted of the following subunit combinations: (A) NR1a/NR2A, (B) NR1a/NR2B, (C) NR1b/NR2A and (D) NR1b/NR2B. Con-G (20 μ M) was applied as indicated by the horizontal line in the inset, while NMDA/glycine were applied throughout the experiment. Recordings were obtained with transfected HEK293 cells voltage-clamped at -70 mV, pH 7.35, at 25°C .

A

S2 (a)
 E⁶⁵⁷FVD QV^TGLSDKKF QRP^HD^YSPPF RFGTVPNGST ERNIRNNY^PY MHQ^YM^TRFN^Q RGV^EDALVSL KTGKLD⁷²⁶
 E⁶⁵⁸YVD QV^SGLSDKKF QRP^ND^FSPPF RFGTVPNGST ERNIRNNY^AE MH^AY^MG^KFN^Q RGV^DDALLSL KTGKLD⁷²⁷

S2 (b)
 A⁷²⁷FIY DAAVLN^YKAG RDEGCKLVTI GSG^VI^FFASTG YGIA^LQK^SP WKRO^IDLA^LL QFVGDGEMEE LE^TILWLTGIC HNEKNEVMSS QLDI⁸¹⁴
 A⁷²⁸FIY DAAVLN^YMAG RDEGCKLVTI GSG^KV^FFASTG YGIA^IQK^DSG WKRO^VDLA^LL QLFGDGEMEE LE^AILWLTGIC HNEKNEVMSS QLDI⁸¹⁵

Fig. 3. Inhibition of NR1b/NR2A-containing NMDAR ion currents by con-G. (A) Amino acid sequences of the S2 regions of rat NR2A (top row; residues 657–814) and rat NR2B (bottom row; residues 658–815) NMDAR subunits. Amino acids that are different in NR2A and NR2B are highlighted in red. (B–E) Inhibition by con-G (20 μM) of NMDA (100 μM)/glycine (10 μM)-induced ion flow through the channels of NR1b combined with different NR2A/2B chimeric receptors as labeled on the graphs. Con-G was applied during the times indicated by a horizontal line in the inset, and NMDA/glycine were applied throughout the experiments. Recordings were obtained with transfected HEK293 cells voltage-clamped at -70 mV, pH 7.35, at 25°C .

Fig. 4.

Inhibition of NMDAR ion currents by con-G. (A–C) Inhibition of NMDA/glycine-induced ion flow through the channels of different NMDARs containing NR1b and point mutants of NR2A as labeled on the graphs. (D–G) Inhibition by con-G of NMDA/glycine-induced ion flow through ion channels containing NR1b and the indicated variant NR2B subunits. Different NMDA concentrations were chosen for NR2B variants in order to achieve the same level of activation (~ 90%) as for wild-type NR1b/NR2B. Glycine concentrations were fixed at 10 μM, and were applied throughout the measurements. Con-G (20 μM) was applied as indicated by the horizontal line in the insets of each panel. Recordings were obtained with transfected HEK293 cells voltage-clamped at -70 mV, pH 7.35, at 25°C.

Fig. 5.

The effect of NR2B-S1/S2 chimeras on the inhibition of NMDARs by both con-G and con-T. (A) Inhibition by 20 μ M con-G and of inward current flow, produced by 100 μ M NMDA/10 μ M glycine, in ion channels consisting of NR1b + NR2B compared to (B) the inhibition observed in NR1b + NR2B[2A-S2] NMDARs by 20 μ M con-G. (C) Inhibition of NR1b + NR2B or (D) NR2B[2A-S2] under these same conditions. Electrophysiological recordings were obtained with transfected HEK293 cells voltage-clamped at -70 mV, pH 7.35, at 25°C . (E) K_i values for con-G and con-T to NMDARs consisting of NR1b subunits in combination with NR2B or NR2A/NR2B chimeric subunits.

Fig. 6.

Dose-response relationships for NMDA and NR1b + NR2A/NR2B chimeric subunits. (A) The whole-cell currents from different cells were normalized to the response produced by 100 μM NMDA in the presence of 10 μM glycine. The response at 500 μM NMDA was set at 100%. The EC_{50} for NMDA obtained with NR1b/NR2B was $21.4 \pm 3 \mu\text{M}$. (B) The EC_{50} -NMDA values for all mutated variants. The NR2A and NR2B subunits and their variants are designated on the illustration in combination with NR1b in all cases. Cells were voltage clamped at -70 mV , pH 7.35, 25°C . The data for NR1b/NR2A and NR1b/NR2B were taken from (Sheng et al., 2007).

Fig. 7. Inhibition of NMDAR ion currents by ifenprodil. (A,B) Inhibition by ifenprodil (20 μ M) of NMDA (100 μ M)/glycine (10 μ M)-induced ion flow through the channels of NR1b combined with (A) NR2B and (B) NR2B[2A-S2]. Because of the lower sensitivity of NR2A to ifenprodil, the concentration of the inhibitor was raised to 120 μ M in the cases of (C) NR2A and (D) NR2A[2B-S2]. The percent inhibition by ifenprodil under the conditions of (A–D) is graphically represented in panel (E).

Fig. 8.

Voltage-dependent whole cell NMDA-activated currents in NMDAR-transfected cells. In A–D, the normalized whole cell currents (pA) of the cells are plotted against their holding potentials (mV). (○) Mg^{2+} is absent from the extracellular solution. (▲) the extracellular solution contained 100 μM $MgCl_2$. The subunit compositions of the transfected NMDARs are indicated in each panel.

Fig. 9. Crystal structure of NR1(S1–S2)/NR2A(S1–S2) domains (Furukawa et al., 2005) showing the backbone positions of the two amino acid residues of interest, K⁷³⁸ and Y⁷⁵⁴.

Table 1

on-G inhibition of current flow in NMDARs containing NR1b + variant NR2 subunits

NMDAR composition NR1b +):	τ_{off} (sec)	k_{on} ($10^3 \text{ M}^{-1}\text{sec}^{-1}$)	$K_{\text{i}}\text{-[con-G]} k_{\text{off}}/k_{\text{on}}$ (μM)	inhibition (%) ^a
NR2A	nr	nr	nr	nr
NR2B	102 ± 37^b	12.5 ± 2.6^b	0.8 ± 0.4^b	96 ± 3^b
NR2A[2B-S2]	80 ± 15	13.3 ± 1.7	0.94 ± 0.21	95 ± 4
NR2A[2B-S2(a)]	nr	nr	nr	nr
NR2A[2B-S2(b)]	76 ± 18	11.9 ± 2.1	1.11 ± 0.33	93 ± 3
NR2A[K ⁷³⁸ M]	70 ± 10	6.4 ± 0.5	2.2 ± 0.4	85 ± 5
NR2A[Y ⁷⁵⁴ K/I ⁷⁵⁵ V]	3.5 ± 0.4	15.8 ± 1.4	18.1 ± 1.9	53 ± 5
NR2A[L ⁷⁶⁵ I/G ⁷⁶⁸ D/P ⁷⁷⁰ G]	nr	nr	nr	nr
NR2A[I ⁷⁷⁵ V/L ⁷⁷⁹ I/F ⁷⁸² L/V ⁷⁸³ F]	nr	nr	nr	nr
NR2A[T ⁷⁹³ A]	nr	nr	nr	nr
NR2A[Y ⁷⁵⁴ K]	3.3 ± 0.3	14.3 ± 1.2	21.2 ± 1.8	50 ± 5
NR2A [I ⁷⁵⁵ V]	nr	nr	nr	nr
NR2A[K ⁷³⁸ M/Y ⁷⁵⁴ K]	66 ± 12	9.1 ± 0.6	1.7 ± 0.3	90 ± 4
NR2B[2A-S2]	1.4 ± 0.2	12.4 ± 2.3	57.6 ± 7.2	40 ± 5
NR2B[M ⁷³⁹ K]	0.9 ± 0.2	14.5 ± 3.2	76.6 ± 12.9	33 ± 4
NR2B[K ⁷⁵⁵ Y]	35 ± 5	14.9 ± 1.8	1.9 ± 0.3	92 ± 3
NR2B[M ⁷³⁹ K/K ⁷⁵⁵ Y]	1.1 ± 0.2	14.2 ± 2.6	64.0 ± 9.7	38 ± 5

^aThe percentage of the steady state current inhibited by con-G. nr, no response.^bData from (Sheng et al., 2007).