Whole cell voltage clamp recordings were performed to assess the ability of conantokin-G (con-G), conantokin-T (con-T), and a 17-residue truncated form of conantokin-R (con-R[1–17]) to inhibit N-methyl-D-aspartate (NMDA)-evoked currents in human embryonic kidney 293 cells transiently expressing various combinations of NR1α, NR1b, NR2α, and NR2B receptor subunits. Con-T and con-R[1–17] attenuated ion currents in cells expressing NR1α/NR2α or NR1α/NR2B. Con-G did not affect NMDA-evoked ionic currents in cells expressing NR1α/NR2A, but it showed inhibitory activity in cells expressing NR1α/NR2B receptors and the triheteromeric combination of NR1α/NR2A/NR2B. An Ala-rich con-G analog, con-G[Q6G/S16A/N17A] (Ala/con-G, where γ is Gla), in which all nonessential amino acids were altered to Ala residues, manifested subunit specificity similar to that of con-G, suggesting that the replaced residues are not responsible for selectivity in the con-G framework. A sarcosine-containing con-T truncation analog, con-T[1–9] containing receptor; con, conantokin; Gla, γ-carboxyglutamic acid (Gla); Fmoc, 9-fluorenylmethoxycarbonyl.

The N-methyl-D-aspartate receptor (NMDAR) is a ligand-gated ion channel that participates in a number of neurophysiological processes including learning and memory, and it has been implicated in many neuropathological disorders, such as Alzheimer’s disease, epilepsy, and ischemic cell death. The genes encoding the subunits that comprise the multicompartment complex fall into two major families. The NR1 family consists of a single gene that yields eight different isoforms (NR1α–h) arising from alternative splicing (1, 2). The NR2 family consists of four separate genes, each encoding a separate protein (NR2A–D) (3). Further, a NR3 subunit, with at least two splice variants (NR3-1 and NR3-2), has been identified (4). In mammalian systems, functional channels are formed upon the coassembly of NR1 and NR2 subunits (5) and require the coagonists glycine and glutamate for activation (6, 7). NR1 and NR3-1 expression is ubiquitous, whereas NR2 and NR3-2 expression is developmentally and regionally specific (4, 8). Hence, the particular subunit combinations that are present at certain times or locations govern a variety of pharmacological properties of the NMDAR (9), such as the response to agonists, antagonists, and/or inhibitors.

Conantokins are small (17–27 residues) neuroactive peptides derived from the venoms of marine snails of the genus Conus. The three major peptides in the conantokin family identified to date are conantokin (con-G), con-T, and con-R. These peptides contain γ-carboxyglutamic acid (Gla) residues (10, 11), possess a high degree of α-helicity (12), and bind divergent cations (12–14). Pharmacologically, conantokins act by selectively inhibiting the NMDAR (10, 15, 16). Recently, these peptides have gained therapeutic importance because of their ability to function as anticonvulsants (17) and as anti-Parkinsonian agents (18), and they have provided neuroprotection within therapeutically acceptable times in a rat model of transient focal brain ischemia (19).

Emerging evidence suggests that conantokins behave as subunit-selective NMDAR antagonists. A recent report demonstrated that when combined with NR1, con-R is selective for NR2A and NR2B, with minimal effects on NR2C and NR2D (17). Another study showed that con-G is selective for NR2B-containing receptors (20). No reports have been documented which reveal con-T subunit selectivity. Thus, despite the high degree of sequence and structural homology among the conantokins, diversity exists with respect to their action at the NMDAR, depending on receptor subunit composition. Because of this, we sought to explore further the ability of wild-type and
variant conantokins to inhibit NMDA-evoked currents in cells transiently expressing various NMDAR subunit combinations. The results presented herein provide insights into the mechanism of conantokin-receptor interactions and the subunit specificity of these interactions.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—The amino acid sequences of the conantokins that were synthesized herein and used for the studies described in this report are provided below:

Con-G: NH2-GEYyGGKAMAAvLARv3v-NH2

Con-T: NH2-GExyYKMKLYNLrA3vEvvKNK2v-NH2

Con-[T–9/G1Src/Q6G]: (CH3)NH-GEYyYKMKLYNLrA3vEvvKNK2v-NH2

Con-[R–17]: NH2-GEYyYKMKLYNLrA3vLARv3v-NH2

Solid phase peptide synthesis was employed throughout using an Applied Biosystems (Foster City, CA) model 435A peptide synthesizer. All methods were as described previously (12, 14). Fmoc strategy was used with the following side chain protecting groups: O-Bu for Asp, Glu, Glu, Ser, 14 and Tyr; Trt for Asn, and Cys; Pmc for Arg; and Boc for Lys. Fmoc-derivatized Glu was synthesized as described previously (21). The peptides were synthesized as the peptide amides, using PAL resin (PerSeptive Biosystems, Framingham, MA). Peptides were deprotected on the resin using trifluoroacetic acid (TFA) (O-trifluoroacetylpropylsilane/dithiothreitol (44:2.5:1:2.5, v/v/v/w) for 3 h at room temperature. All peptides were purified further by FPLC anion exchange chromatography using BiosecadeAEAA20 (Bio-Rad) resin. The materials were then desalted on a Sephadex G-15 column (1.5 × 100 cm), equilibrated, and eluted with 0.1% NH4OH. The peptides were characterized further by reverse-phase high performance liquid chromatography and matrix-assisted laser desorption/ionization-time of flight mass spectrometry as described (22).

**Expression of Recombinant NMDA Receptors**—cDNAs encoding the rat NR1a, NR1b, NR2A, and NR2B receptors (subcloned into pRC/CMV or pRK5) were kindly provided by Dr. David R. Lynch (23). Plasmid pEGFP-N1, encoding a red shifted variant of wild-type green fluorescent protein, was purchased from CLONTECH Laboratories, Inc. (San Diego).

HEK293 cells were transiently transfected using the calcium phosphate precipitation method (24). Briefly, the cells were grown to ~50% confluence on 35-mm polystyrene-coated dishes and transfected with combinations of NR1- and NR2-containing plasmids (5 μg of DNA/dish) along with pEGFP-N1 for identification of positive cells. Plasmid ratios for different combinations were 1:1:3 for green fluorescent protein, NR1, and NR2, respectively, and 1:1.5:1.5 for cells coexpressing the NR1a, NR2A, and NR2B trimeric combination. Following transfection, the cells were maintained in fresh medium containing 500 μM ketamine to prevent NMDA-mediated cell death. Electrophysiological recordings were performed within 24–48 h post-transfection.

**RESULTS**

**Conantokin-G Is an NMDAR Subunit-selective Antagonist**—Con-G, con-T, and con-R[1–17], the last a carboxy-terminal truncated variant of con-R which retains wild-type activity (14), were assessed for their ability to inhibit NMDA-evoked currents in HEK293 cells expressing recombinant NMDAR subunits. Although IC50 values for conantokins determined in electrophysiological assays are in the range of 0.1–1 μM (17, 26, 27), their slow onset of inhibition makes these agents difficult to study at low micromolar concentrations because of the time required to reach equilibrium. To achieve a more rapid inhibition, each of the various peptides (50 μM) was used to determine the rate of onset (τon), offset (τoff), and percentage of inhibition in cells expressing NR1a/NR2A or NR1a/NR2B. The values are summarized in Table I for results obtained at NR1a/NR2A receptors and in Table II for results obtained at NR1a/NR2B receptors. As illustrated in Fig. 1, A and B, con-T and con-R[1–17] inhibited NMDA-evoked currents (>95%) in receptors formed at both combinations of subunits. The con-T-based 9-mer, con-T[1–9/G1Src/Q6G], also inhibited at both subunit forms. On the other hand, con-G inhibited currents in cells expressing NR1a/NR2B but had little to no effect on cells expressing NR1a/NR2A. Similarly, the alanine-rich con-G analog, Ala-con-G, paralleled this subunit specificity.

The course for the onset of peptide inhibition (τon), of ~5 s, as well as for offset of inhibition (τoff), of ~2 min, was similar for all parent peptides at their active subunit combinations. These values correlate with results obtained using cultured neurons (26). The Ala/con-G peptide manifested a τon similar to con-G. However, the con-T-based 9-mer inhibited 3-fold faster at both NR1a/2A and NR1a/2B combinations compared with con-T.

**The Alanine Acid in Position 5 Contributes to Conantokin Inhibition and Selectivity**—The three conantokins under investigation share a high degree of amino-terminal sequence homology, maintaining identity of the first four residues (GEγγ). The residue at position 5, which is essential for activity as determined by alanine replacement studies (27–29), is different in each species and is represented by a Tyr, Leu, and Val in con-T, con-G, and con-R, respectively. Variant conantokins containing conservative mutations at this position, in which a residue from one peptide was replaced by a residue native to another peptide, were tested to address the importance of this amino acid for activity and subunit specificity. The degree of inhibition as well as the τon and τoff values were determined using a single peptide concentration of 50 μM. The values for results obtained at NR1a/NR2A receptors and NR1a/NR2B receptors are summarized in Tables I and II, respectively.

Con-T variants with Val, Phe, or Trp substitutions at sequence position 5 were reasonably well tolerated at receptors containing NR1a/NR2B subunits, whereas the Val and Trp derivatives were much less effective at NR1a/NR2A-containing receptors (Table I). Con-T[5Y5L] was completely inactive at concentrations up to 100 μM in the [3H]MK-801 assay and, therefore, was not tested in this electrophysiological study.

The most dramatic effects occurred in con-G by substituting Leu5 with a Tyr, the residue present at that location in con-T. This analog, in contrast to wild-type con-G, inhibited (70.6 ± 3.7%) currents in cells expressing NR1a/NR2A (Fig. 2A). Further, for this variant, it was observed that the data describing the rate of onset was best fit using a second order exponential. This resulted in fast (τfast = 0.48 ± 0.08 ms) and slow (τslow = 4.1 ± 0.9 s) components, where the slow component was com-
TABLE I
Conantokin inhibition at recombinant NR1a/NR2A receptors expressed in HEK293 cells

| Peptide | $\tau_{on}$ | $\tau_{off}$ | $k_{on}$ | Inhibition | $IC_{50}$ MK-801 | $k_{off}/k_{on}$ | n |
|---------|-------------|--------------|---------|------------|----------------|----------------|---|
| Con-T   | 4.2 ± 0.5   | ~30          | 4.07    | 92.9 ± 4.5 | 0.35           | 8.2            | 5 |
| YSF     | 2.5 ± 0.5   | <15          | 6.61    | 77.0 ± 13.4| 0.95           | 10.1           | 4 |
| YSV     | <1          | <1           | 20.0    | 27.5 ± 3.9 | 6.7            | 50             | 4 |
| con-G   | ND          | ~45          | 14.4 ± 6.6 | 1.7 | ND | 3 |
| 1-9/G15Src/Q66 | 1.4 ± 0.3 | 6.5 ± 1.3 | 10.8 | 93.2 ± 4.5 | 0.48 | 1.4 | 6 |
| Con-G   | NA*         | NA           | NA      | 0          | 0.48           | NA             | 10 |
| L5Y     | 4.1 ± 0.9   | 5.3 ± 1.3    | 1.18    | 70.6 ± 3.7 | 13.0           | 158            | 7 |
| L5V     | 0.48 ± 0.07 | 38.3         | 38.3    | 4.88       |                |                |    |
| L5I     | NA          | NA           | NA      | 0          | 0.54           | NA             | 4 |
| Ala/con-G | ND        | ND           | ND      | 26.1 ± 2.5 | 0.15           | ND             | 3 |
| Con-R[1–17] | 5.5 ± 1.2 | ~60          | 3.31    | 91.9 ± 6.7 | 0.90           | 5.0            | 8 |
| V5L     | 3.1 ± 0.5   | ~30          | 5.85    | 90.1 ± 4.5 | 0.84           | 5.97           | 7 |
| V5Y     | 2.2 ± 0.5   | ~75          | 8.95    | 98.4 ± 1.6 | 1.09           | 1.49           | 4 |
| V5I     | 3.6 ± 0.5   | 3.4 ± 0.9    | 3.4     | 65.9 ± 9.5 | 2.0            | ND             | 4 |

* Each peptide was tested at a concentration of 50 µM in the presence of 100 µM NMDA and 10 µM glycine. Values are the mean ± S.E. for the specified number of cells (n). Sre, sarcosine.

$^b$ $k_{on}$ was determined from the relationship $k_{on} = k_{on}[\text{conantokin}] + k_{off}$ where $k_{on} = 1/\tau_{on}$ and $k_{off} = 1/\tau_{off}$.

$^c$ $IC_{50}$ values obtained for inhibition of $[^{3}H]$MK-801 binding as determined in previous studies are included for comparison.

$^d$ ND, not determined.

$^*NA$, not applicable.

TABLE II
Conantokin inhibition at recombinant NR1a/NR2B receptors expressed in HEK293 cells

| Peptide | $\tau_{on}$ | $\tau_{off}$ | $k_{on}$ | Inhibition | $IC_{50}$ MK-801 | $k_{off}/k_{on}$ | n |
|---------|-------------|--------------|---------|------------|----------------|----------------|---|
| Con-T   | 5.7 ± 1.2   | ~60          | 3.20    | 94.9 ± 4.5 | 0.35           | 5.3            | 4 |
| YSF     | 4.5 ± 0.5   | ~30          | 3.78    | 92.9 ± 3.3 | 0.95           | 8.7            | 5 |
| YSV     | 3.4 ± 0.4   | 6.6 ± 1.0    | 2.67    | 71.5 ± 3.1 | 6.73           | 57             | 6 |
| Y5W     | 7.9 ± 0.7   | ~15          | 0.5     | 61.5 ± 2.7 | 1.7            | 200            | 3 |
| 1-9/G15Src/Q66 | 1.9 ± 0.6 | 3.3 ± 0.6 | 4.8     | 94.3 ± 4.5 | 0.48           | 6.2            | 8 |
| Con-G   | 4.7 ± 1.2   | >120         | 3.80    | 91.3 ± 5.5 | 0.48           | 2.3            | 7 |
| L5Y     | 1.8 ± 0.2   | 3.4 ± 0.5    | 4.28    | 73.6 ± 6.6 | 13.0           | 67             | 5 |
| L5I     | 3.6 ± 0.3   | ~120         | 5.55    | 84.6 ± 2.4 | 0.84           | 5.4            | 6 |
| V5Y     | 3.8 ± 0.5   | ~75          | 4.34    | 87.4 ± 3.1 | 1.73           | 7.6            | 5 |
| Ala/con-G | 6.0 ± 0.9 | ND          | 3.2     | 97.5 ± 3.5 | 0.15           | 2.6            | 3 |
| Con-R[1–17] | 5.0 ± 1.3 | >120        | 3.83    | 88.4 ± 4.4 | 0.09           | 2.2            | 5 |
| V5L     | 3.7 ± 0.5   | ~45          | 4.56    | 85.9 ± 3.1 | 0.84           | 4.9            | 4 |
| V5I     | 4.0 ± 0.6   | ~60          | 4.67    | 97.9 ± 3.6 | 0.09           | 3.6            | 4 |
| V5I     | 3.8 ± 0.3   | 8.9 ± 1.9    | 2.78    | 70.7 ± 12.2| 2.0            | 40             | 4 |

* Each peptide was tested at a concentration of 50 µM in the presence of 100 µM NMDA and 10 µM glycine. Values are the mean ± S.E. for the specified number of cells (n). Sre, sarcosine.

$^b$ $k_{on}$ was determined from the relationship $k_{on} = k_{on}[\text{conantokin}] + k_{off}$ where $k_{on} = 1/\tau_{on}$ and $k_{off} = 1/\tau_{off}$.

$^c$ $IC_{50}$ values (µM) obtained for inhibition of $[^{3}H]$MK-801 binding as determined in previous studies are included for comparison.

$^d$ ND, not determined.

parable to wild-type con-T and con-R[1–17]. Con-G[L5Y] also inhibited at NR1a/NR2B-containing receptors (Fig. 2B); however, the degree of inhibition was lower than wild-type con-G (73.6 ± 6.6%), and the $\tau_{off}$ was nearly 40-fold faster (~3 s). Further replacement of Leu* in con-G with the closely related Ile or Val led to a retention of inhibitory activity in receptors composed of NR1a/NR2B (Fig. 2, lower) but had no inhibitory effect on NR1a/NR2A-containing receptors (Fig. 2A).

Replacement of Val in con-R[1–17] with a Tyr or Leu did not alter the extent of inhibition greatly (~90%) at either subunit combination tested, whereas replacement with Ile slightly lowered (to ~70%) this parameter. The $\tau_{off}$ was significantly faster for con-R[1–17/V5I] (~10 s) than for the V5I (~45 s) or V5Y (~60 s) con-R variants. The extents of inhibition of these variants correlated with the potency determined in $[^{3}H]$MK-801 assay, where V5Y > wild-type V5 > V5I.

Exon 5 in the NR1 Subunit Influences Conantokin Inhibition—Exon 5 encodes a 21-amino acid insert in the extracellular N-terminal region of the NR1 subunit (residues 190–211). Alternative splicing of this exon (where NR1a denotes the absence of exon 5 and NR1b denotes the presence of exon 5) influences receptor sensitivity to modulators such as Zn$^{2+}$, polyamines, and protons (30). Because conantokins have been proposed to act allosterically at the polyamine site (15), HEK293 cells were transfected with NR1b/NR2A and NR1b/ NR2B to determine whether the inhibitory effects of the conantokins displayed any specificity for this splice variant. In contrast with their null effects at NR1a/NR2A recombinant receptors, con-G and Ala/con-G displayed some degree of inhibition (~40%) at NR1b/NR2A-containing receptors (Fig. 3) and completely inhibited NR1b/NR2B-containing receptors (Table III). Insofar as the incomplete inhibition noted with con-G and Ala/con-G at the NR1b/NR2A receptor form may reflect either weak affinity or partial antagonism, the effect of 100 µM peptide was also examined. A higher degree of inhibition occurred (~60%) for a 100 µM application of con-G and Ala/con-G (data not shown). This concentration response indicates weak affinity, not partial antagonism, at the NR1b/NR2A combination. Con-G[L5Y] also inhibited NMDA-evoked currents at NR1b/ NR2A (~75%) with properties similar to those observed at NR1a/NR2A; however, the L5V and L5I variants of con-G were inactive in this regard (Fig. 3). Con-T and con-R[1–17] com-
Completely inhibited NMDA-evoked currents in both of these subunit combinations (Table II). The \( \tau_{on} \) of inhibition was faster by at least 2-fold for all wild-type peptides at NR1b- versus NR1a-containing receptor combinations. The \( \tau_{off} \) of inhibition was somewhat slower for all peptides at NR1b/NR2B receptors than NR1a/NR2B. All peptides tested at NR1b/NR2B receptors displayed apparent \( K_d \) values of less than 1 \( \mu \)M. Although these values must be considered only a first approximation of the true binding constants, they demonstrate that conantokins have a 5–10-fold increase in affinity at receptors containing NR1b compared with those containing NR1a.

**Conantokin Inhibition at Triheteromeric Receptors**—Several reports support the existence of triheteromeric receptors comprised of NR1/NR2A/NR2B in both native and recombinant systems (31–33). Furthermore, this subunit combination displays sensitivity to various modulators, such as Zn\(^{2+}\) and ifenprodil, which are intermediate between the effects observed at NR1/NR2A and NR1/NR2B (34, 35). Because con-G is active at NR1a/NR2B receptors, but not those containing NR1a/NR2A, cells were transfected with NR1a, NR2A, and NR2B (1:1.5:1.5 ratio) to evaluate whether con-G displays similar intermediate effects (Table II).

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**Fig. 1. Con-G is selective for the NR2B subunit.** Con-G, con-T, con-R[1–17], and Ala/con-G were tested at a concentration of 50 \( \mu \)M to assess their ability to inhibit NMDA-evoked currents in HEK293 cells transiently expressing NR1a/NR2A (panel A) or NR1a/NR2B (panel B). Currents were elicited by the addition of 100 \( \mu \)M NMDA and 10 \( \mu \)M glycine followed by an intermittent application of peptide solution as indicated by solid bars. The time course of onset (\( \tau_{on} \)) and offset (\( \tau_{off} \)) of inhibition was determined by fitting the data using a single exponential. For slowly dissociating peptides, the rate of offset (\( \tau_{off} \)) was estimated by measuring currents evoked at 30-s intervals during a wash period after removal of peptide, plotting the data against time, and fitting using a single order exponential. The percent inhibition and time course values are presented in Tables I and II.
con-G and Ala/con-G (50 μM) are capable of inhibiting NMDA-evoked currents in cells transfected with all three subunits. The representative traces show complete inhibition; however, the degree of inhibition by con-G varied from cell to cell (68.2 ± 15.1%, n = 13). Based on the extent of inhibition, the data were divided into at least two populations: 86 ± 2 (n = 7) and 58 ± 3 (n = 6). The rate of offset was also variable, where some cells displayed a fast τoff (~1 s), and others showed a slower τoff (~30 s); yet, there was no correlation between these rates and the degree of inhibition.

Con-T and con-R[1–17] were fully active at this subunit combination and had kinetic values similar to those in the diheteromeric receptor combinations (Table III). Inhibition of current flow by 10 μM ifenprodil was tested for comparison (Fig. 4) and displayed intermediate effects, supporting the coassociation of all three subunits.

**DISCUSSION**

It is demonstrated herein that con-G displays specificity for the NMDAR subunit NR2B, whereas con-T and con-R[1–17] do
PDGF, 50 μM con-G (responses from two separate cells), Ala/con-G, and 10 μM ifenprodil in HEK293 cells coexpressing NR1a, NR2A, and NR2B (1:1:5.1.5 ratio). Currents were elicited by the addition of 100 μM NMDA and 10 μM glycine followed by an intermittent application of peptide solution as indicated by solid bars. Con-T and con-R completely inhibit receptors containing NR1a/NR2A/NR2B.

**NR1a/NR2A/NR2B**

![Peptide traces](image)

**TABLE III**

Conantokin inhibition at recombinant NR1b containing receptors and NR1a/NR2A/NR2B expressed in HEK293 cells

| Peptide$^a$ | $\tau_{on}$ | $\tau_{off}$ | $k_{on}$ | Inhibition $k_{off}$ | $\mu M$ | $n$ |
|------------|-------------|-------------|---------|----------------------|--------|-----|
| NR1b/NR2A  |             |             |         |                     |        |     |
| Con-T      | 1.8 ± 0.2   | -30         | 10.3    | 98.0 ± 2.0           | 3.2    | 4   |
| Con-G      | 0.57 ± 0.10 | 0.93 ± 0.10 | 13.8    | 37.4 ± 10.7          | 77.5   | 6   |
| L5Y        | 3.8 ± 0.6   | 6.0 ± 0.7   | 2.00    | 77.2 ± 11.4          | 83.5   | 5   |
| Ala/con-G  | 0.94 ± 0.07 |           | 56.7    | 98.9 ± 6             |        |     |
| Con-R[1–17]| 1.4 ± 0.1   | 2.2 ± 0.4   | 4.86    | 40.5 ± 10.5          | 98.6   | 9   |
| NR1b/NR2B  |             |             |         |                     |        |     |
| Con-T      | 1.4 ± 0.3   | >120        | 14.3    | 100                  | 0.58   | 5   |
| Con-G      | 1.1 ± 0.2   | >120        | 17.7    | 100                  | 0.47   | 7   |
| Ala/con-G  | 1.6 ± 0.2   | >180        | 12.7    | 100                  | 0.44   | 4   |
| Con-R[1–17]| 2.2 ± 0.4   | >120        | 8.79    | 100                  | 0.95   | 4   |
| NR1a/NR2A/NR2B |       |             |         |                     |        |     |
| Con-T      | 3.2 ± 0.8   | -30         | 5.60    | 100                  | 5.95   | 4   |
| Con-G      | 3.0 ± 1.1   | 0.7 ± 0.2   | 6.09    | 68.2 ± 15.1          | 13     |     |
| Ala/con-G  | >30 (slow)  | -30         | 5.05    | 59.0 ± 14.7          | 3.30   | 4   |
| Con-R[1–17]| 4.2 ± 0.5   | -60         | 4.49    | 98.6 ± 1.4           | 3.70   | 4   |

$^a$ Each peptide was tested at a concentration of 50 μM in the presence of 100 μM NMDA and 10 μM glycine. Values are the mean ± S.E. for the specified number of cells (n).

$^b k_{on}$ was determined from the relationship $k_{obs} = k_{on}[\text{conantokin}] + k_{off}$, where $k_{obs} = 1/\tau_{on}$ and $k_{off} = 1/\tau_{off}$.

Although a high degree of sequence homology and structural similarity exists among the three conantokins, differences among them confer distinct NMDAR inhibitory properties. Because the amino acid located at sequence position 5 differs for these peptides, and this residue has been shown to be essential for activity (14, 27, 29), variant peptides containing variations at this position were used to address the importance of this amino acid for activity and subunit specificity.

In general, it was found that substitutions at position 5 of con-G and con-T led to activity losses for receptors specifically containing the NR2A subunit, but those at con-R were equally well tolerated between NR2A- and NR2B-containing receptors. None of the sequence position 5 variant peptides was as effective as its parent wild-type peptides, except for con-R[1–17]/V5Y. Although con-G[L5Y] is capable of inhibiting current flow in ion channels of NR1a/NR2A receptors, it is a less efficacious inhibitor than wild-type con-G when used with NR1a/NR2B, NR1b/NR2B, and NR1b/NR2A receptors, and it has a faster $\tau_{on}$ (~40-fold) at NR1a/NR2B. These results indicate that this con-G variant, although possessing broader subunit selectivity than con-G, has a lower affinity than wild-type con-G or con-T for NR2B at diheteromeric receptors containing NR1a and either NR2A or NR2B. Additionally, the biphasic nature of the $\tau_{on}$ values for con-G[L5Y] at NR2A-containing receptors suggests an altered mode of binding than is the case for con-G or con-T.

These studies also revealed that residues native to position 5 in the conantokins are not interchangeable with respect to activity at recombinant NMDA receptors. To illustrate, in contrast to con-G(L5Y), con-G(L5V) was ineffective at NR1a/NR2A receptors. Con-T was inactive when Tyr$^5$ was replaced by Leu (29), and less efficacious at NR1a/NR2A and NR1a/NR2B when con-T[V5Y] was employed. Although con-G[L5Y] is not active with NR1a/NR2A or NR1b/NR2A receptor forms, this peptide variant does retain activity at NR1a/NR2B. However, in the con-R background, the native Val at position 5 yields a peptide that is highly active at all tested subunit combinations. These results, coupled with the observation that position 5 substitutions, including Leu, were best tolerated in con-R[1–17], suggest that residues downstream of residue 5 impart broad specificity to con-R[1–17], while tailoring the selectivity of con-G and con-T. The lack of similar effects among the three
conantokin species, with respect to variation in the amino acid at sequence position 5, strongly suggests that other residues within the peptides contribute to affinity, efficacy, and selectivity. Two additional peptides examined in this study have provided some insight into the participation of amino acids other than those residing at sequence position 5. The extensively modified Ala/con-G peptide parallels the subunit selectivity of con-G. This excludes Gla residues at sequence positions 7, 10 and 14, as well as Gln\(^6\), Asn\(^8\), Lys\(^{15}\), Ser\(^{16}\), and Asn\(^{17}\) from functioning as selectivity determinants in the con-G platform. The truncated con-T-based 9-mer inhibits similarly at NR1a/NR2A and NR1a/NR2B receptors, indicating that the residues in con-T which impart indiscriminate subunit reactivity are embodied within the first 9 residues of the peptide.

In the absence of direct structural data on conantokin-NMDA receptor interactions, it is difficult to predict the influence of exon 5 of NR1 (present in NR1b, absent in NR1a) on conantokin inhibition. The fact that con-G and Ala/con-G can inhibit current flow with the NR1b/NR2A-containing receptor, but not with the NR1a/NR2A combination, raises the possibility that the conantokin-NMDA interaction occurs at the interface between NR1 and NR2, in the vicinity of the 21-amino acid insert corresponding to exon 5. Direct interactions between con-G and the region encoded by exon 5 are excluded because all peptides are active at NR1a-containing receptors, especially con-G when NR1a is coexpressed with NR2B. It is possible that the presence of exon 5 may result in a receptor conformation that exposes a cryptic con-G binding locus on the NR2A subunit. Because the peptide \(\tau_{\text{off}}\) value is faster for wild-type peptides at NR1b-containing receptors, and the rate of offset is slower when this subunit is expressed with NR2B, a more accessible conantokin binding site(s) may form. Furthermore, the binding site arising in NR1b/NR2A is less discriminating among peptides because con-G and Ala/con-G display activity, although with weaker affinity due to the fast \(\tau_{\text{off}}\) values for these peptides. It has been suggested that interactions between the extracellular portions of NR1 and NR2 are responsible for differences observed in agonist affinity (36, 37), and this same consideration may also apply to conantokin affinities. It is possible that all three peptides may be acting at the same locus, but the NR1a/NR2A configuration does not accommodate con-G. Accordingly, con-T and con-R may share an identical binding site because they are active at all subunit combinations, but con-G may bind to an overlapping site.

The exact subunit composition and stoichiometry of the NMDAR has not been determined. Several reports have provided evidence for the existence of at least three different subunits (i.e., NR1/NR2A/NR2B) within a single receptor molecule in both native and recombinant systems (31–33). In the current study, cotransfection of NR1a/NR2A/NR2B produced receptors that were completely inhibited by con-T and con-R[1–17], similar to the effects observed using diheteromeric receptors. On the other hand, the NR2B-selective con-G produced results intermediate to the effects observed at diheteromers, as the degree of inhibition ranged from 40–90%. The partial inhibition by con-G at the triheteromeric combination is similar to the response effected by ifenprodil, an NR2B-selective inhibitor (35). These results are consistent with the existence of receptors containing all three subunits. Moreover, these data provide additional evidence that con-G may be acting at the NR1/NR2 interface. Because the exact subunit stoichiometry is yet uncertain, several possibilities exist. When the number of NR2B subunits is higher than the number of NR2A subunits, a greater extent of inhibition occurs, and vice versa. It cannot be excluded that diheteromers of NR1/NR2A and NR1/NR2B are forming within the same cell and thus producing the observed results. However, it has been reported that only 8% of NR1/NR2A and 27% of NR1/NR2B receptors are present in adult rat cortex, whereas the majority of receptors contain at least one of each subunit (32). Therefore, it is likely that each of these combinations exists and that con-G is fully active at NR1a/NR2B, inactive at NR1a/NR2A, and partially active at NR1a/NR2A/NR2B.

The importance of the overall secondary structure of the conantokin is somewhat unclear. In the presence of divalent cations, all three species assume a highly \(\alpha\)-helical structure. However, \(\alpha\)-helicity does not appear to be essential for activity as demonstrated by the data obtained using Ala/con-G, which only contains 8% helicity yet functions similarly to wild-type con-G. Furthermore, con-T and con-G are capable of inhibiting \([\text{MK-801}]+\) binding to adult rat brain sections in the presence of EDTA (38), and an array of conantokin variants lacking helicity maintains potent activity in the \([\text{H}]\text{MK-801}\) binding assay (14, 27). Yet, this does not imply that overall structure is not important for activity. Because little is known regarding the direct binding of conantokins to the NMDAR, it is possible that this interaction may induce secondary structure in the peptides.

It has been reported that con-G acts competitively at the NMDA site (20). There is also evidence that the Glu/NMDA binding site is located on homologous regions of the NR2 subunits (39). Thus, it would follow that conantokins are interacting at the Glu/NMDA recognition site. However, if con-G is acting exclusively at the same site as NMDA, it should be active at NR2A, as it is at NR2B. In general, NMDA competitive antagonists display affinity for recombinant receptors, in the order: NR2A > NR2B > NR2C > NR2D (37, 40, 41). Whereas con-T and con-R inhibit similarly at NR2A and NR2B receptors, con-G clearly has a higher affinity for NR2B. Con-G appears to be the only natural product competitive NMDA site antagonist that displays complete subunit selectivity.

In conclusion, these results provide insight into the molecular features associated with conantokin-selective properties. The present data corroborate previous findings that con-G is a selective NMDAR antagonist. Furthermore, the amino acid in position 5 plays a key role in subunit specificity. The fact that there is not complete reciprocity with respect to the essential position 5 amino acid among the three species of conantokin peptides indicates that there are other downstream residues important for selectivity/activity, which may directly interact with the receptor or impart a conformation necessary for activity. In light of recent reports demonstrating the neuroprotective effects of conantokin in focal ischemia and seizure models (19), the subunit specificity of con-G (20), as well as the results presented herein, these peptides are proving to be extremely useful NMDAR antagonists. Their ability to serve as effective templates for therapeutic modeling and also as tools for probing NMDAR structure and modulation affords these peptides considerable attention. Further, because the combinations of subunits that constitute the functional NMDAR show developmental and regional specificity, inhibition of the NMDAR ion channels by the conantokins exhibits parallel specificity. This suggests that selective inhibitory efficiencies for different conantokins may be exploited to favor conditions or locations where particular receptor subunit combinations are expressed.

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The Amino Acid Residue at Sequence Position 5 in the Conantokin Peptides Partially Governs Subunit-selective Antagonism of Recombinant N-Methyl-d-aspartate Receptors
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