

In vitro selection of peptides acting at a new site of NMDA glutamate receptors

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Oligomeric N-methyl D-aspartate receptor (NMDAR) in brain is a ligand-gated ion channel that becomes selectively permeable to ions upon binding to ligands. For NMDAR channel, the binding of glutamate and glycine results in opening of the calcium permeable channel. Because the calcium influx mediated by NMDAR is important for synaptic plasticity and excitotoxicity, the function of NMDA receptors has been implicated in both health and disease. Native NMDA receptors are thought to be heteromeric pentamers with a central ion conduction pathway. There are five genes (NR1, 2A, 2B, 2C, and 2D) encoding various subunits that have been cloned, and NR1 is thought to be the essential subunit since it forms a functional channel by itself. To study NMDAR structure and function, we have searched for peptide modulators of NR1 using random peptide bacteriophage libraries. The peptides were identified based on their specific association with a purified receptor fusion protein that contains the putative ligand binding domain. We report the identification of one group of cyclic peptides (Mag-1) with a consensus sequence of CDGLRHMWFC. Using biochemical binding analysis and patch clamp electrophysiological recording, we show that the synthetic Mag-1 peptides cause noncompetitive inhibition of the receptor channel activity.

Keywords: NMDA, peptide display, neurotoxicity

N-methyl D-aspartate receptor (NMDAR) channels belong to the superfamily of ligand-gated ion channels. The glutamate-mediated channel activity is important for synaptic plasticity, synaptogenesis, and excitotoxicity¹⁻⁵. NMDARs are encoded by at least five genes (NR1, 2A, 2B, 2C, and 2D) and their spliced variants³. NR1 is an essential subunit for mediating glutamate-induced channel activity, since a homomultimeric NR1 complex, when expressed in *Xenopus* oocytes, exhibits some of key properties found in native channels^{5,6}. These properties include direct permeability of calcium, voltage dependent Mg²⁺ blockade of the ion channel, and binding sites for modulators such as Zn²⁺, glycine, and polyamines^{5,7-13}. The other subunits by themselves cannot form a functional channel; however, they coassemble with NR1 to form heteromultimers that increase the functional expression and channel diversity. The NR1 polypeptide can be divided into two regions: an extracellular aminoterminal domain containing the putative agonist binding site, and a hydrophobic core region with multiple transmembrane segments that participate in the formation of the ion conducting pathway^{5,6}. Although several classes of compounds have been found to modulate the NMDAR, at the molecular level little is known regarding the amino acid residues that are responsible for the interaction. To better understand the physiological roles of NMDARs with different subunit composition, it would be helpful to develop specific ligands that are capable of distinguishing different subunits.

Ligands or toxins that modulate ion channel activity play an instrumental role in our current understanding of structure and

function of ion channels. We have used a random peptide display strategy to identify peptides with potential biological activity. The peptide libraries used in this study were constructed by inserting random oligonucleotides into the gene that encodes bacterial filamentous phage major coat protein pVIII, a strategy that has first been used for pIII¹⁴⁻¹⁷, a minor coat protein. Thus, the sequence of expressed peptide as a part of pVIII coat protein is "bar-coded" by DNA sequence inside the viral particle. Each phage particle in the pool carries a distinct peptide on its surface. With several libraries consisting of more than 10 billion different peptides, and an NMDAR fusion protein as a target, we have identified multiple peptide families that specifically recognize the extracellular aminoterminal domains of the NR1 subunit. We describe one class of peptides that we isolated from a constrained cyclic library (CX₃C), consisting of 0.5 × 10¹⁰ independent clones. These peptides bind specifically to NR1 and cause noncompetitive inhibition of the channel activity.

Results

Peptide isolation. To obtain the purified "target" protein for isolating specific peptide ligands, the putative aminoterminal domain of NR1 (N-NR1, a.a 1-561^{5,6}) was subcloned and stably expressed in Chinese hamster ovary (CHO) cells as a fusion protein (Fig. 1A), in which the carboxyl-terminal portion contains an epitope tag for monoclonal antibody (Mab179) and a signal for a glycosylphosphatidylinositol (GPI) anchor¹⁸. The surface-expressed N-NR1

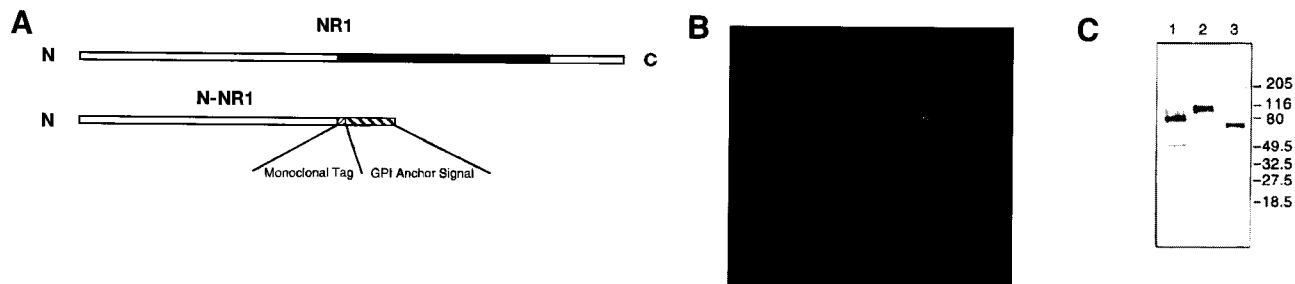


Figure 1. Expression of the extracellular aminoterminal domain of NR1. **(A)** Schematic representation of the NR1 subunit and N-NR1 fusion protein. Black box indicates the transmembrane core region consisting of multiple membrane-spanning segments. The N-NR1 (a.a. 1-561) was expressed with a "tail" peptide containing a tag for monoclonal antibody (Mab179) and GPI signal. **(B)** Immunohistochemical detection of the surface-expressed N-NR1. A stable CHO cell line (2G8) with high expression of N-NR1 was selected by fluorescence-activated cell sorting (FACS). The surface expression of N-NR1 was detected by Mab179 followed by Texas-red labeled secondary antibody (Vector Labs, CA). **(C)** Posttranslational modification of N-NR1. The N-NR1 fusion protein expressed in CHO (2G8) cells was detected by immunoblot using Mab179 followed by peroxidase-conjugated secondary antibody. The binding was visualized using the Enhanced Chemoluminescence (ECL, Amersham). Lane 1: total cell lysate; lane 2: Surface N-NR1 after cleavage by PI-specific phospholipase C; lane 3: PIPLC-cleaved N-NR1 after digestion of endo-F.

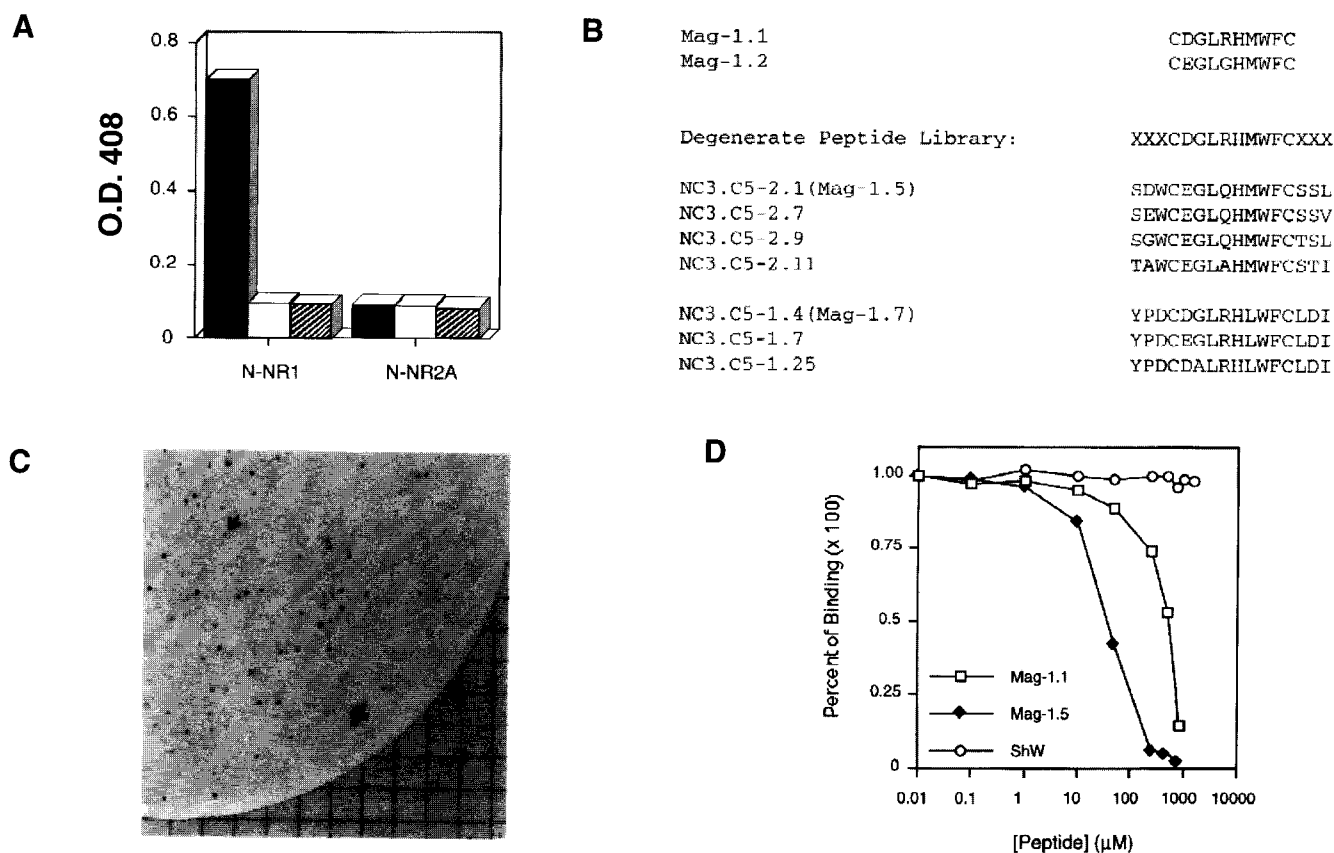


Figure 2. Isolation and affinity maturation of Mag-1 peptides that associate with N-NR1. **(A)** Specific association of Mag-1.1 with N-NR1. After five rounds of panning, phagemid supernatants of individual clones in the enriched pool were collected for phage ELISA assay to determine their specificity. One clone (Mag-1.1) that specifically binds N-NR1 is shown. Dash bars: wells coated with bovine serum albumin (BSA) only; white bars: BSA plus Mab179; black bars: BSA, Mab179 plus the captured N-NR1 (left) or N-NR2A (right). The phage binding was detected by rabbit antiphage antibody. **(B)** Sequence comparison of Mag-1 peptides. Two clones (Mag-1.1 and Mag-1.2) were isolated from initial screen. Based on the Mag-1.1 sequence, a degenerate library was constructed. The high-affinity clones in this enriched pool were further selected by a filter binding assay (see C for examples). Deduced amino acid sequences for seven of the 50 clones were aligned and revealed two related classes of peptides that are homologous to Mag-1.1. **(C)** Identification of clones with higher affinity. A mutagenesis library was constructed containing 4×10^8 Mag-1 derived clones. After three rounds of the affinity panning in the presence of 500 μ M synthetic Mag-1.1, the resultant pool was plated and the peptide-pVIII fusion proteins were immobilized on nitrocellulose filters. Colonies with strong (big arrow) or weak (small arrow) binding were identified. **(D)** Binding of Mag-1 related peptides. The ability of synthetic Mag-1 peptides to compete with the Mag-1.1 phage binding to N-NR1 fusion protein was determined by phage ELISA. The vertical axis indicates the relative binding compared to the signal obtained without adding the peptide inhibitors. The horizontal axis indicates logarithmic concentration of the peptides added in μ M: open squares (\square) for Mag-1.1 (GGGCDGLRHMWFCGGG); closed squares (\blacksquare) for Mag-1.5 (GGGSDWCEGLQHMWFCSSLGGG); open circles (\circ) for ShW, an unrelated peptide (GGEDRQHRKKQGG). The experiments were performed in triplicates and the average values are shown. Background binding was determined and subtracted using the purified N-NR2A protein.

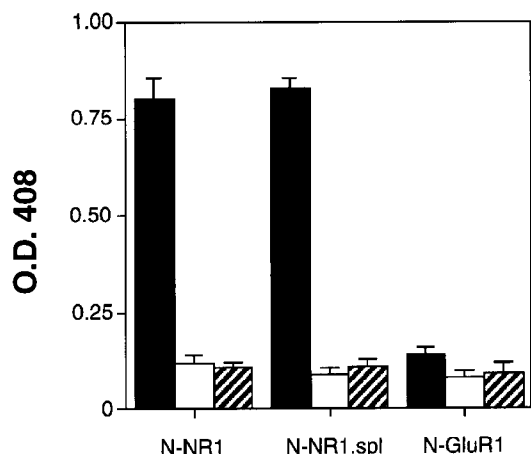


Figure 3. Binding specificity of Mag-1 peptides to amino-terminal domains of glutamate receptors. Phage ELISA experiments were carried out using N-NR1, N-NR1.spl, and N-GluR1. Dash bars: wells coated with bovine serum albumin (BSA) only; white bars: BSA plus Mab179; black bars: BSA, Mab179 plus the captured fusion proteins for N-NR1, N-NR1.spl, or N-GluR1 (as indicated). Experiments were carried out in triplicate. Error bars indicate the standard deviation.

fusion protein was released from the cell membrane by PI-specific phospholipase C (PIPLC) cleavage resulting in a polypeptide with a molecular weight of approximately 96 kD (Fig. 1B, C). This recombinant N-NR1 is glycosylated and sensitive to endoglycosidase F (Endo F) digestion (Fig. 1C, lane 3), similar to what was found for the native NR1¹⁹.

Peptide libraries used in this study were constructed by inserting random oligonucleotides in frame and amino-terminal to the pVIII gene of bacteriophage ϕ 1, which encodes the major coat protein, permitting the display of peptides on the surface of the phage particles. The sequence of the displayed peptide was deduced from the nucleotide sequence within the virion. To identify the phage clones (or peptides) that specifically associate with N-NR1, we mixed the affinity-purified N-NR1 with a constrained octamer random peptide library "CX₈C" that has eight random amino acid residues flanked by two cysteines and a complexity of approximately 0.5×10^{10} clones. The displayed peptides presumably are constrained by intramolecular disulfide bonds, and the binding of phage particles to the immobilized N-NR1 is likely through multivalent associations because each phage particle carries more than 100 copies of the peptide. After five consecutive rounds of selection and amplification (Fig. 2, legend), clones that bind specifically to the immobilized N-NR1 fusion protein were identified by testing the binding of individual phage clone to the immobilized N-NR1 (phage ELISA)²⁰ (Fig. 2A). One family of phage clones (Mag-1.1 and Mag-1.2) was identified with the deduced sequences of CDGLRHMWFC and CEGLRHMWFC. To determine the specificity for NR1, the Mag-1.1 clone was also tested for binding to amino-terminal domain of NR2A (N-NR2A, a.a. 1-527), an accessory subunit that coassembles with NR1 in vivo. The N-NR2A was cloned and expressed in the same vector used for N-NR1. Mag-1.1 binds to N-NR1 but not N-NR2A (Fig. 2A).

To increase binding affinity of the identified peptides, a mutagenized peptide library was constructed to cover 4×10^6 independent clones derived from Mag-1.1 sequence. The peptides displayed in this library have degenerate sequences of Mag-1.1 plus six additional random residues flanking the two cysteines (Fig. 2B). Screening the mutagenized library in the presence of 500 μ M of

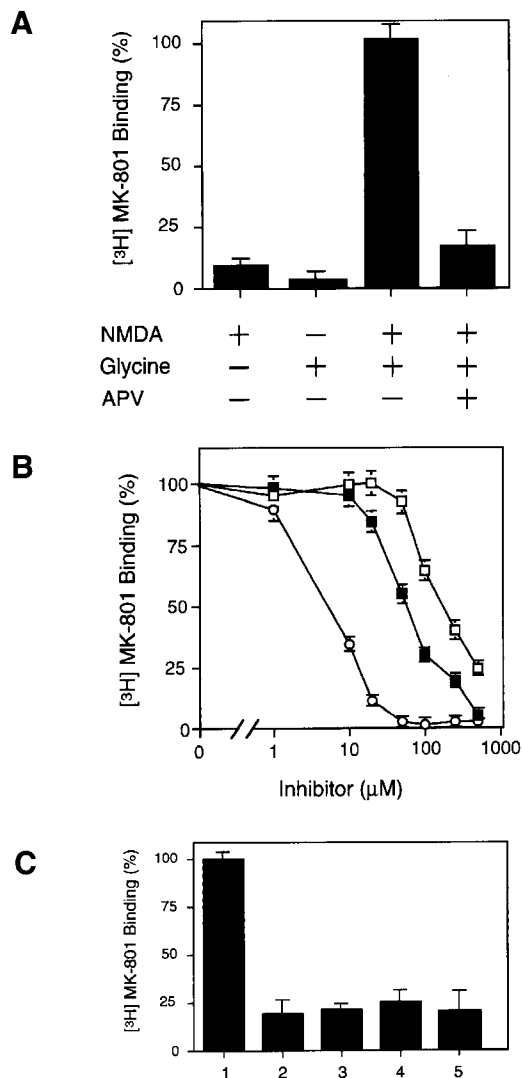


Figure 4. Inhibition of [³H]-MK-801 binding by Mag-1 peptides. (A) Membrane preparations from total rat brain were used to detect the [³H]-MK-801 binding. NMDAR-specific [³H]-MK-801 binding was determined in the presence of different combinations (indicated at the bottom of the graph) of 20 μ M NMDA, 10 μ M glycine, and 100 μ M DL-APV. The binding assays were carried out in triplicate and normalized against the signal obtained in the presence of NMDA and glycine. The error bars indicate standard deviation. (B) Mag-1 peptides inhibit NMDAR-specific [³H]-MK-801 binding. The [³H]-MK-801 binding experiments were carried out in the presence of DL-APV (\circ); Mag-1.1 (\square); and Mag-1.5 (\blacksquare). The binding signals were quantified and normalized against [³H]-MK-801 binding in the absence of peptides. Vertical axis indicates the normalized [³H]-MK-801 binding; the horizontal axis indicates the final concentrations of competitors in μ M. (C) Noncompetitive inhibition by Mag-1.5 peptide. [³H]-MK-801 binding to rat brain membrane was induced by 20 μ M NMDA and 10 μ M glycine (1). The binding was inhibited in the presence of 250 μ M Mag-1.5 (2). This inhibition was challenged by glycine (3), glutamate (4), or glutamate plus glycine (5) at final concentration of 1 mM. All experiments were done in triplicate and error bars indicate the standard deviation.

the synthetic Mag-1.1 peptide resulted in a pool with an improved affinity (Fig. 2B). The higher affinity clones within the pool were further selected by bivalent receptor binding to the pVIII-peptide fusion proteins immobilized on nitrocellulose filters, where intensity of signal (Fig. 2C, arrows) often correlates with affinity. Using this bivalent filter binding assay, we were able to screen thousands

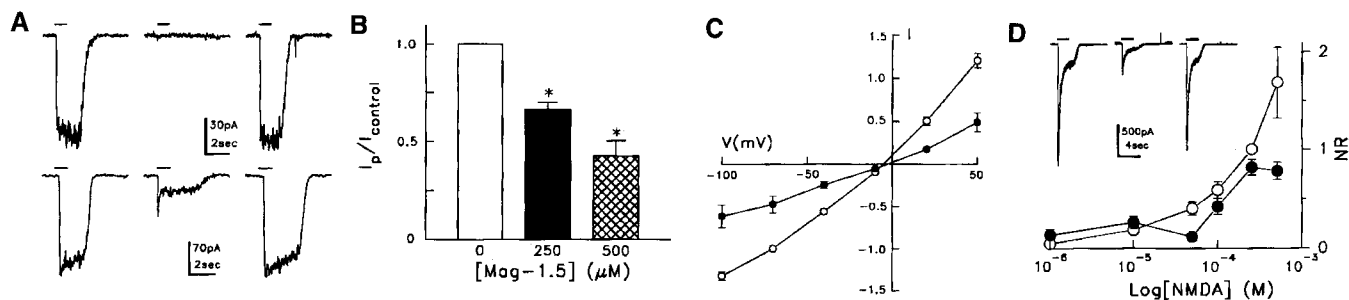


Figure 5. Mag-1 related peptides inhibit the NMDA-induced currents in transfected HEK293 cells. (A) Upper panel: Transfected HEK 293 cells show NMDA-induced responses which are sensitive to APV. Whole-cell voltage clamp recordings were obtained from the transfected HEK 293 cells. Puff application of 50-μM NMDA induced an inward current at a holding potential of -70 mV (left trace). Coapplication of 100 μM DL-APV with 50 μM NMDA abolished the NMDA-induced current (middle trace) with the recovery shown in the right trace after APV was removed. Lower panel: Inhibition of NMDA-induced currents in transfected HEK 293 cells. Whole-cell recording was carried out as described above. The 50 μM NMDA-induced current (left trace) was reduced by coapplication of 500 μM Mag-1.7 (middle trace). The recovery from the Mag-1.7 mediated inhibition was shown in the right trace. Bars above traces indicate the duration of NMDA or NMDA +Mag-1.7 application. **(B) The inhibition of NMDAR current by Mag-1.7 is dose-dependent.** Normalized peak current responses are illustrated. The experiments were carried out at a holding potential of -70 mV. Control response is the current evoked by 50 μM NMDA and 10 μM glycine. Coapplication of 250 μM (n = 3) or 500 μM (n = 10) Mag-1.7 with the control NMDA (50 μM) significantly reduced the NMDA-induced current, which is indicated by * ($p < 0.05$, student test). **(C) The inhibition of NMDAR current by Mag-1.7 is not voltage-dependent.** The 50 μM NMDA-induced currents (○) and the currents evoked by NMDA plus 500 μM Mag-1.7 (●) were normalized using records obtained at a holding potential of -70 mV. Data were collected and averaged from four cells. Error bars represent standard error. **(D) The inhibition of NMDA-induced currents by Mag-1.5 is noncompetitive to NMDA.** Whole-cell voltage clamp recordings were carried out at a holding potential of -70 mV. The peak currents that were evoked by different concentrations of NMDA in the presence (●) or absence (○) of 300 μM Mag-1.5 are normalized according to the current induced by 250 μM NMDA. Data were averaged from three to six cells. Error bars represent standard error. **Insert:** An example showing the effect of 300 μM Mag-1.5 on 500 μM NMDA-induced current. The left trace is the current induced by 500 μM NMDA. Addition of 300 μM of Mag-1.5 reduced the current shown in the middle trace, with the recovery shown on the right. The holding potential was -70 mV. Bar above each trace represents the duration of NMDA or NMDA plus Mag-1.5 application.

of clones conveniently and avoid the potential bias introduced by growth competition in liquid culture. To directly compare the relative binding of these peptides, phage binding in the presence of synthetic peptides of Mag-1.1 and Mag-1.5 was carried out. The results indicate that Mag-1.5 is approximately tenfold more potent than Mag-1.1 in competing with binding of the phage particle (Fig. 2D).

Comparison of amino acid sequences has revealed significant homology between NMDAR1 and non-NMDA glutamate receptor GluR1 within their amino-terminal domains²¹. To further test the binding specificity of the Mag-1 related peptides, we expressed the amino-terminal domains of GluR1 (a.a. 1-431, N-GluR1) and a spliced variant of NMDAR1 (a.a. 1-584, N-NR1.spl), which carries an insertion of 21 amino acids in the amino-terminal domain and exhibits different sensitivity to Zn²⁺ modulation²². The N-GluR1 and N-NR1.spl were expressed in the same vector used for NR1 to produce fusion proteins with a monoclonal tag at the COOH-terminus to aid affinity purification. Phage ELISA experiments were done to compare the binding specificity of N-NR1, N-GluR1, and N-NR1.spl to the phage clone of Mag-1.5 (Fig. 3). Both N-NR1 and N-NR1.spl bind to Mag-1.5. In contrast, under the same conditions, no binding can be detected for N-GluR1. Together, these results suggest that Mag-1.5 is capable of distinguishing the NH₂-terminal domains of NMDAR from that of a non-NMDA glutamate receptor, GluR1.

Properties of the identified peptides. The channel activity of NMDAR is modulated by several classes of compounds including glutamate, glycine, Mg²⁺, Zn²⁺, polyamines, MK-801, and possibly conantokin-G, a peptide toxin isolated from predatory marine snail of *C. geographus*^{5,7-13,23,24}. At the molecular level, little is known about the sites to which these modulators bind. To determine whether the Mag-1.1 peptide recognizes any of these known sites, we tested the binding of Mag-1.1 phage to the N-NR1 in the presence of the modulators. As expected, Mg²⁺ and MK-801 do not compete with Mag-1.1 phage binding to N-NR1 because their binding sites are likely to be located in the ion pore aligned by several subunits^{24,25}. In addition, we found that the Mag-1.1 bind-

ing was not sensitive to 10 mM of glutamate, glycine, spermidine, Zn²⁺, or 10 μg/ml of conantokin-G (data not shown), suggesting that the Mag-1 peptides recognize a new site.

MK-801, an anticonvulsant, binds to the native NMDAR channel only in its opened configuration²⁴. Ligands that modulate NMDAR channel activity result in changes in [³H]-MK-801 binding to the receptor in membranes prepared from rat brain¹³. The [³H]-MK-801 binding is induced by a combination of NMDA and glycine, indicating that it is NMDAR-specific binding (Fig. 4A). If the binding of Mag-1 peptides to the N-terminal domain of NR1 perturbs channel activity, one may observe changes in [³H]-MK-801 binding to the native receptor. To test the potential modulatory activity of Mag-1 peptides, high performance liquid chromatography (HPLC) purified synthetic peptides containing an intramolecular disulfide bond were used in [³H]-MK-801 binding experiments. In the presence of 500 μM of Mag-1.1 peptide the binding of [³H]-MK-801 is significantly reduced and the same concentration of Mag-1.5 peptide eliminated [³H]-MK-801 binding (Fig. 4B). This suggests that the binding of Mag-1 peptide inhibits the channel activity and causes a decrease of [³H]-MK-801 binding. The potency of the inhibition is consistent with the results from competition phage ELISA (Fig. 2D). The half maximal inhibition (IC₅₀) of Mag-1.5 on [³H]-MK-801 binding was estimated to be approximately 45 μM. In addition, we observed no changes for the Mag-1.5 mediated inhibition in the presence of glycine, glutamate, or glutamate plus glycine (Fig. 4C). This result further supports the idea that Mag-1.5 peptide is acting at a site other than those for glycine and glutamate.

To directly test the peptide effects on cloned NMDAR channel activity, NR1 and NR2A were coexpressed in human embryonic kidney (HEK) cell line 293 by transient transfection²⁶⁻²⁸. The channel activity, monitored by patch clamp recording using a fast perfusion system⁷, has similar properties to native channels, as judged by glycine potentiation, voltage sensitive Mg²⁺ blockade, and inhibition of competitive and noncompetitive antagonists such as 2-amino-5-phosphonovalerate (APV) and MK-801^{7-12,23,24}. In the presence of 500 μM of Mag-1.7, which has an apparent

affinity similar to Mag-1.5, the NMDA-induced channel activity was reduced by more than 50% at -70 -mV holding potential. This inhibition by Mag-1.7 peptide is reversible, since the channel activity was fully recovered after a brief wash (Fig. 5A). The inhibitory effect by Mag-1.5 was dose-dependent (Fig. 5B) and voltage insensitive as similar degree of inhibition was observed at different holding potentials from -100 mV to $+50$ mV (Fig. 5C). As for the $[^3\text{H}]$ -MK-801 binding studies (Fig. 4), increased NMDA concentration does not reverse the block by Mag-1.5 peptides (Fig. 5D). Combining this result and those from the competition binding (Fig. 4), Mag-1 peptide appears to recognize a novel site on N-NR1, and to cause noncompetitive inhibition of NMDA receptor channels.

Discussion

Excitotoxic mechanisms have a well-established role in the pathogenesis of neuronal injury following acute CNS insults such as ischemia and trauma²⁹⁻³². Increasing evidence now supports their roles in chronic neurodegenerative disorders such as motor neuron diseases³²⁻⁴¹. Although the molecular mechanisms underlying excitotoxic neuronal injury are still being elucidated, a large body of evidence indicates that the cascade of events resulting from elevation of intracellular free calcium is likely to play a major role, which is thought to be mediated by NMDA receptor and voltage-gated calcium channels^{31,42}. NMDA receptor has several very important properties of relevance to excitotoxicity, particularly, the ability to permeate calcium ions. Thus, understanding of NMDAR properties at the molecular level and development of reagents that specifically modulate these receptors may be of importance for understanding excitotoxicity-caused disease and providing potential leads that could facilitate future treatments. With further improvements in the affinity and stability of Mag-1 related peptides, it would be of great interest to test Mag-1 family of peptides for potential neuronal protective activity.

We showed that the binding of the Mag-1.1 peptide is not affected by the known NMDAR modulators, including glutamate, glycine, Mg^{2+} , Zn^{2+} , polyamines, or MK-801. In addition, because the recording for our electrophysiological experiments was not done under reducing condition, it is unlikely that the oxidized synthetic Mag-1.7 peptide interacts with the reported redox sites implicated in NMDAR⁴³. This is further supported by the fact that the channel activity can be fully restored by a brief wash to remove Mag-1.7 peptide (Fig. 5A). Together, the Mag-1 peptides discussed here are a group of noncompetitive NMDAR antagonists that recognize a putatively new site embedded in the extracellular N-terminal domain of NR1.

Native NMDARs are likely to be pentameric complexes consisting of NR1 and other subunits such as NR2A. Using the N-NR1, a truncated protein containing putative agonist binding domain of NR1 subunit, we have identified novel peptides with modulatory activity on native NMDAR. Although the purified N-NR1 receptor is not a pentamer (data not shown), it is clear that some of the important structural features of NR1 are retained in the fusion protein, suggesting a strategy that may be applicable to other ion channels and transporters. Perhaps additional rounds of the affinity maturation using the reported approach could result in peptides with even higher modulatory activity. Together, our results suggest the feasibility of isolating biologically active peptides for ligand-gated ion channels through *in vitro* selection, an approach that could have significant pharmacological applications.

Experimental protocol

Expression and biochemical analysis. Coding sequence for N-NR1 (a.a. 1-561), N-NR2A (a.a. 1-527), N-NR1.spl (a.a. 1-584), and N-GluR1 (a.a. 1-431) were obtained by PCR amplification, cloned into eukaryotic cell

expression vector driven by the SR α promoter, and confirmed by DNA sequencing. It was then fused in frame to a nucleotide fragment coding for "AAACLEPYTACDLA PPAGTTDAAHPGRSVV PALL PLLAGTLLLLLETATAP", where the underlined fragment represents the tag for Mab179 and the remaining portion is a GPI anchor signal sequence¹⁸. The stable transfectants were identified after 2 to 4 weeks of G418 selection (1 mg/ml). Clones with higher expression were isolated based on the fluorescence intensity and isolated using a FACS-STAR plus (Becton Dickinson, San Jose, CA). Immunohistochemical detection was carried out using Mab179 (0.5 $\mu\text{g}/\text{ml}$) followed by 15 $\mu\text{g}/\text{ml}$ of secondary antibody (Vector Labs, Burlingame, CA). Imaging was performed according to the protocol provided by the manufacturer. Immunoblot detection was carried out using a protocol previously described⁴⁴. The Endo-F digest was performed in the presence of 0.05% sodium dodecylsulfate (SDS) for 4 h at 37°C. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Prestained molecular weight markers (Bio-Rad, Hercules, CA) are indicated.

Peptide library construction. The filamentous phage pVIII gene was chemically synthesized using four overlapping oligonucleotides. This was inserted into the NheI and HindIII sites of the phagemid vector pBAD⁴⁵, which places the expression of the pVIII gene under the control of the araB promoter. The resulting plasmid was designated p8V2. A cloning site consisting of two noncomplementary BstXI sites was included at the 5' end of gene VIII, and this site was used to clone collections of degenerate oligonucleotides using the half-site strategy as previously described¹⁷.

Peptide screening. Six wells of a 96-well plate were precoated with Mab179 (5 $\mu\text{g}/\text{well}$) at 37°C for 1 h, blocked with 1% bovine serum albumin (BSA) for 30 min at 37°C. The N-NR1 fusion protein was first released by PIPLC digest after extensive washes of cells that express N-NR1. The released N-NR1 protein in the MEM medium was affinity-purified by Mab179 capture on the plates from 100 μl of N-NR1 (0.1 $\mu\text{g}/\text{ml}$) at 22°C for 1 h. The phage binding was carried out by incubating with a total of 10^{12} tu (transducing units) of a CX₂C library with a complexity of 0.5×10^{10} recombinants. Binding was carried out in buffer containing 10 mM Tris-HCl (pH 8.1), 100 mM NaCl and 1 mM EDTA for 3 h at 4°C. Unbound material was removed with three washes of the binding buffer. The bound phagemid particles were recovered by adding 100 μl of 0.1 M glycine-HCl (pH 2.2). After 10 min elution at room temperature, the eluates were immediately neutralized by mixing with an equal volume of 0.1 M Trisbase (pH 9.2). The recovered phagemids were amplified in bacteria¹⁷, and resultant phage preparations were used for the following round of purification. After five consecutive rounds of selection, the ELISA tests were performed using the supernatant of overnight cultures in the presence of 100 mM Hepes-NaOH (pH 7.5) and 100 mM NaCl. The wells were coated with receptors according to the procedure described above. The bound phage particles were first detected by using rabbit antiphage antibody (1:10,000) followed by alkaline phosphatase (AP)-conjugated secondary antibody (1:5000, Sigma, St. Louis, MO). After washing the wells with the binding buffer, 100 ml of developing solution containing 1 mg/ml pNPP (Sigma) in 10 mM diethanolamine-HCl (pH 9.7) was added. The alkaline phosphatase activity was monitored at the wavelength of 408 nm using a microtiter plate reader. For affinity maturation, an oligonucleotide was synthesized to encode the following amino acid sequence: XXXCDGLRHMWFCXXX, where Xs indicate random amino acids; C represents cysteines; and underlined letters indicate positions that have 90% probability of having indicated residues and 10% of the rest of residues. This oligo was inserted into the phagemid vector for library construction as described¹⁷. The panning was performed using a procedure identical to that described above except that the immobilized N-NR1 was first incubated with 500 μM Mag-1.1 peptide for 30 min prior to addition of the library. After three rounds of selection, approximately 5,000 clones of the enriched pool were plated on LB plates supplemented with 50 $\mu\text{g}/\text{ml}$ of ampicillin. After 10 h of incubation at 37°C, bacterial colonies were transferred on a nitrocellulose filter. The immobilized bacteria were lysed by chloroform vapor. After blocking with 5% nonfat milk, the filters were incubated with a premixed solution containing 10 $\mu\text{g}/\text{ml}$ of N-NR1 fusion protein and 1 $\mu\text{g}/\text{ml}$ Mab179 for 2 h at 4°C. The binding of the bivalent receptor to immobilized peptide fusion proteins was detected by secondary antibody (1:5,000) and visualized using AP substrates (BCIP and NBT from Bio-Rad). Fifty colonies with higher intensity were selected. The single-strand DNA was prepared according to standard phagemid preparation protocol. Their sequences were determined using a

sequenase kit (Amersham, Arlington Heights, IL) according to procedures provided by the manufacturer. The peptides used in the study were chemically synthesized and oxidized to form intramolecular disulfide bonds. The resultant material was HPLC-purified and the purity/composition was confirmed by mass spectroscopy.

[³H]-MK-801 binding assay. Total rat brains (Charles River, Wilmington, MA) were homogenized in 0.32 M sucrose using a Polytron homogenizer. The homogenate was centrifuged at 1000 x G for 10 min. The resultant supernatant was centrifuged again at 48,000 x G for 20 min. The remaining steps of membrane preparation, including extensive washes, were carried out according to the protocol by Wong et al.¹³. The final pellets were resuspended in 5 mM Tris (pH 7.4) (0.1 g starting tissue weight per 1 ml of buffer). The binding of (+)-[³H]-5-methyl-10,11-dihydro-5H-dibenzol[a,d]cyclohepten-5,10-imine maleate ([³H]-MK-801) (NEN, 29 Ci/mmol) was performed as described by Wong et al.¹³ except that all reactions were done at 23°C for 60 min in the presence of 20 μM NMDA, 10 μM glycine, and 10 nM of [³H]-MK-801 and competitors at the indicated concentrations. The reactions were stopped by filtration over polyethyleneimine-soaked GF/B filters. Nonspecific binding was determined in the presence of 10 μM MK-801.

Electrophysiological recording. Whole-cell recordings were obtained from HEK 293 cells transfected with pRc/CMV vectors (Invitrogen, San Diego, CA) containing either NMDAR1 or NMDAR2A cDNA. Transfection was done by calcium phosphate precipitation with a total of 20 μg of plasmid DNA (6 μg of NMDAR1 and 14 μg of NMDAR2A) per 10-cm plate. At 10 to 16 h after transfection, culture medium without glutamate and aspartate was changed and supplemented with DL-APV (final concentration was 1 mM) to prevent cell death. Recordings were carried out at 36 to 72 h after transfection. Ligand application was controlled by solenoid-mediated switching (NResearch, Newark, NJ) between the two sides of a θ tubing positioned within 50 to 100 mm from the cell. Single isolated cells were lifted from the bottom of the recording chamber immediately after formation of the whole-cell recording configuration to optimize rapid ligand application. During recordings, cells were continuously bathed with a Krebs solution (control) containing: 145 mM NaCl; 5.4 mM KCl; 1.8 mM CaCl₂; 33 mM glucose; 25 mM HEPES; pH was adjusted to 7.4 with NaOH (22°C). Agonist or antagonist was added directly to the control solution, pH was readjusted if necessary. Patch pipettes (4 to 12 MΩ) used for recordings were filled with intracellular solution containing: 140 mM KCl; 1 mM MgCl₂; 1 mM CaCl₂; 10 mM HEPES; 5.5 mM BAPTA; 4 mM MgATP; 2 mM TEA; pH was adjusted to 7.2 with KOH. During the recording, the NMDAR channel was activated by NMDA with the indicated concentrations plus 10 μM glycine. Voltage-clamp currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and stored on a computer (Dell Computer, Austin, TX) for later analysis. Both data acquisition and analysis were done using pCLAMP software (version 6.0, Axon Instruments).

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