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Binding of ArgTX-636 in the NMDA Receptor Ion Channel

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Abstract

The *N*-methyl-D-aspartate receptors (NMDARs) constitute an important class of ligand-gated cation channels that are involved in the majority of excitatory neurotransmission in the human brain. Compounds that bind in the NMDAR ion channel and act as blockers, are use- and voltage-dependent inhibitors of NMDAR activity and have therapeutic potential for treatment of a variety of brain diseases or as pharmacological tools for studies of the neurobiological role of NMDARs. We have performed a kinetic analysis of the blocking mechanism of the prototypical polyamine toxin NMDAR ion channel blocker argitoxin-636 (ArgTX-636) at recombinant GluN1/2A receptors to provide detailed information on the mechanism of block. The predicted binding site of ArgTX-636 is in the pore region of the NMDAR ion channel formed by residues in the transmembrane M3 and the M2 pore-loop segments of the GluN1 and GluN2A subunits. To assess the predicted binding mode in further detail, we performed an alanine- and glycine-scanning mutational analysis of this pore-loop segment to systematically probe the role of pore-lining M2 residues in GluN1 and GluN2A in the channel block by ArgTX-636. Comparison of M2 positions in GluN1 and GluN2A where mutation influences ArgTX-636 potency suggests differential contribution of the M2-loops of GluN1 and GluN2A to binding of ArgTX-636. The results of the mutational analysis are highly relevant for the future structure-based development of argitoxin-derived NMDAR channel blockers.

Graphical abstract

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Introduction

The *N*-methyl-D-aspartate receptors (NMDARs) are ligand-gated ion channels belonging to the ionotropic glutamate receptor (iGluR) superfamily of neurotransmitter receptors. NMDARs are present in the vast majority of excitatory synapses in the central nervous system and couple binding of the amino acid neurotransmitters glycine (Gly) and L-glutamate (Glu) to opening of a cation channel; leading to depolarization and increase in intracellular Ca²⁺ levels of the post-synaptic neurons ¹. In addition to their role in excitatory neurotransmission, NMDAR activity is involved in several forms of neuronal plasticity; including long-term potentiation of synaptic signaling strength that is thought to constitute the cellular basis for learning and memory ². Furthermore, NMDARs are therapeutic targets for treatment of neurological and psychiatric brain diseases that involve dysregulated glutamatergic activity ³. These include Alzheimer's disease, epilepsy, cerebral ischemia, traumatic brain injury and depression : for which NMDAR inhibitors are in current use as approved drugs or undergoing clinical trials ⁴.

The NMDARs are heterotetrameric receptor complexes assembled from two GluN1 and two GluN2 subunits, and are activated by simultaneous binding of Gly to the GluN1 subunits and Glu to the GluN2 subunits, respectively. GluN3 subunits can also assemble with GluN1 and GluN2, but the structure and function of GluN3-containing receptors are currently unresolved. Each subunit contains three semiautonomous domains: the N-terminal domain (NTD), the ligand binding domain (LBD), and the transmembrane domain (TMD) (Fig. 1). In addition, an intracellular C-terminal domain protrudes from the TMD and is the most variable domain among NMDAR subunits. The TMD consist of three membrane-spanning segments (M1, M3 and M4) and a re-entrant M2 segment that connects M1 and M3 (Fig. 1) ⁵. The core of the ion channel in NMDARs is formed by the M2 and M3 segments from the GluN1 and GluN2 subunits, and contains binding sites for a variety of ligands including divalent cations ^{6; 7}, small-molecule organic compounds ^{8; 9; 10} and naturally occurring toxins from spider and wasp venom ^{11; 12; 13}. When bound in the ion channel, these ligands act as channel blockers to sterically hinder ion flux, hereby effectively inhibiting NMDAR signaling activity. Among the various types of NMDAR inhibitors, channel blockers in particular have demonstrated promising therapeutic potential for treatment of a spectrum of brain diseases ^{1; 14; 15}. However, although they all inhibit NMDAR function by steric block of the ion channel, structurally different classes of channel blockers display surprisingly diverse physiological effects. For example, therapeutic use of NMDAR channel blockers such as the anticonvulsant dizocilpine (MK-801) and the dissociative anesthetics

phencyclidine (PCP) and ketamine is limited by side effects such as ataxia, cognitive dysfunction and psychosis^{16; 17}, whereas memantine is well-tolerated and is used for treatment of epilepsy, Parkinson's disease and Alzheimer's disease^{18; 19}. The reason for these differences among NMDAR channel blockers likely reflects kinetic and mechanistic differences in the interactions between these molecules and the ion channel. The ligand-binding mechanism of NMDAR ion channel blockers is considered quite complex^{15; 19; 20}. First, NMDAR channel blockers are use-dependent inhibitors as they can only access their binding sites during receptor activation and ion channel opening. Second, most NMDAR channel blockers are cationic and their binding is thus sensitive to the membrane potential as the ligand experience forces from the membrane electrical field similar to those experienced by permeating cations. Third, NMDARs are subject to voltage-dependent block by endogenous Mg^{2+} , which can influence binding of exogenous pore-binding ligands. Subtle differences in these parameters, even among structurally closely related ion channel blockers, may combine to produce widely different overall blocking kinetics at native NMDARs. Thus, characterization of the molecular, kinetic and mechanistic details of NMDAR channel block for different compound classes is important for future drug development.

Considering their potential as therapeutics, the molecular pharmacology remains surprisingly incomplete for most NMDAR channel blockers. A contributing factor to this has been lack of insight into the molecular structure of the TMD region, including the ion channel, of NMDARs. There is no crystal structure available for an intact NMDAR, but the full-length structure of the closely related GluA2 α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type iGluR provides a template for creation of homology models of NMDARs (Fig. 1)⁵. However, even though the GluA2 structure was determined at an overall 3.6 Å resolution, parts of the TMD region were poorly resolved and is thus not a good template for generating models of the NMDAR ion channel. The core structure of iGluR ion channels is structurally related to Na^+ and K^+ channels²¹ and crystal structures of K^+ channels²² in particular has been used to model NMDAR ion channel structures^{23; 24; 25}. The inner linings of the NMDAR ion channel is formed by equal contribution of the M2 and M3 segments from the GluN1 and GluN2 subunits arranged diagonally (1:2:1:2) to each other around the channel center axis in the receptor complex (Fig. 1C)^{5; 26}. Viewed from the extracellular side, the channel contains an upper cavity (often denoted the outer extracellular vestibule) and a narrow, lower passage that constitute the channel pore and determine ion selectivity and conductance^{20; 27; 28; 29; 30; 31; 32}. Mutational analysis of M2 and M3 segments have been extensively used to identify channel-exposed residues in M2 and M3 as well as residues that are key determinants for binding of channel blockers^{27; 28; 29; 30; 31; 32; 33; 34; 35; 36; 37; 38; 39; 40; 41; 42}.

The prototypical argitoxin ArgTX-636 originates from the venom of the orb weaver spider *Argiope lobata*^{11; 13; 24} and is among the most potent NMDAR channel blockers with affinities in the low nanomolar range^{43; 44}. Most NMDAR channel blockers show little or no selectivity among NMDAR subtypes^{3; 45; 46; 47}, but interestingly ArgTX-636 is more than 20-fold selective for the GluN1/2A and GluN1/2B over GluN1/2C and GluN1/2D NMDAR subtypes^{44; 48}. ArgTX-636 is therefore of interest as a pharmacological tool for the evaluation of the roles of NMDAR subtypes in normal brain function and disease as well

as a potential scaffold for drug discovery. However, despite these noteworthy properties several aspects of the blocking mechanism and molecular binding mode of ArgTX-636 at NMDARs are not well-characterized^{24; 43; 44}. In this work, we explore the mechanism and time-course of block for ArgTX-636 at the NMDAR-subtype GluN1/2A. Moreover, we analyze the role of residues in the M2 segment of GluN1 and GluN2A subunits for ion channel block of ArgTX-636, MK-801 and Mg²⁺. Our results provide a detailed description of the mechanism of block, its kinetics and the voltage-dependency of ArgTX-636 and highlight the differential role of residues in the M2 segment of the NMDAR ion channel for binding of different ion channel blockers.

Results and Discussion

Blocking mechanism of ArgTX-636 at GluN1/2A receptors

Despite previous studies of ArgTX-636 block of NMDAR channels^{24; 43; 44; 49; 50; 51}, there is no detailed information on the kinetics of channel block. We therefore characterized the time-course of ArgTX-636 channel block of recombinant GluN1/2A receptors expressed in HEK293 cells using whole-cell patch-clamp electrophysiology (*Materials and Methods*) (Fig. 2). Using a fast solution-exchange recording protocol, we first analyzed the time-course of onset and recovery of ArgTX-636 block of GluN1/2A currents evoked by Glu (100 μ M) and Gly (50 μ M) using different concentrations of ArgTX-636 (Fig. 2A and 2B). At a holding potential of -80 mV, the macroscopic current decay and recovery produced by ArgTX-636 block and unblock were best described by a single exponential function (Fig. 2A). The rate of channel block ($1/\tau_{\text{block}}$) was linearly dependent on the ArgTX-636 concentration, whereas the rate of channel unblock ($1/\tau_{\text{unblock}}$) was independent of ArgTX-636 concentration (Fig. 2B). These results suggest a one-step bimolecular binding mechanism of ArgTX-636 to the open ion channel as previously also found for other channel blockers of NMDARs^{10; 52; 53}. The association (K_{on}) and dissociation (K_{off}) rate constants of ArgTX-636 with the open channel were $4.4 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $0.36 \pm 0.09 \text{ s}^{-1}$, respectively, as calculated from the measured time constants (Fig. 2B); yielding a K_{B} of 48 nM, which is similar to previously reported IC_{50} values obtained from inhibition of steady-state GluN1/2A currents evoked by saturating agonist concentrations in *X. laevis* oocytes^{24; 44}. Comparison of K_{on} for ArgTX-636 block of the GluN1/2A open channel with those for classical small-molecule NMDAR blockers such as ketamine, MK-801, PCP, and memantine (determined under similar experimental conditions; although mainly at native NMDARs) show that ArgTX-636 binds with an approximately 10-fold higher association rate constant ($4.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) compared to for example memantine which is considered a fast NMDAR blocker with K_{on} of 0.3 to $0.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ^{52; 54; 55}. The time-course of dissociation for synthetic organic small-molecule NMDAR channel blockers vary substantially with MK-801 having an unusually slow unbinding rate (0.002 to 0.03 s^{-1})^{8; 10; 55} to memantine with 100-fold faster unbinding kinetics (0.2 to 0.4 s^{-1})^{18; 54; 55}. Thus, having a dissociation rate constant of 0.36 s^{-1} makes ArgTX-636 a relatively fast unbinding blocker relative to other organic NMDAR channel blockers, with the rank order in terms of unbinding rate being memantine $\sim$ ArgTX $>$ ketamine $>$ PCP $\sim$ MK-801.

Previous work has found that the IC_{50} for steady-state ArgTX-636 block of native and recombinant NMDARs to be strongly dependent of the membrane potential^{24; 56}, which suggests that the association rate of ArgTX-636 with the channel binding site increases and/or the dissociation rate decreases with increasing negative membrane potential. We examined the binding and unbinding rates using the application protocol described in Fig. 2A with an ArgTX-636 concentration of 500 nM at membrane potentials ranging from -40 to -100 mV and determined on- and off-rates for ArgTX-636 channel block (Fig. 2C and D). The rate of onset of block was observed to increase sharply with increasingly negative membrane potential once the membrane potential was below -60 mV; increasing approximately 5-fold per 20-mV negative depolarization of the membrane (Fig. 2D, *left panel*). The dissociation rate was modestly dependent on membrane potential in the -60 to -100 mV range; decreasing less than 2-fold over this range (Fig. 2D, *right panel*). This suggests that the increase in ArgTX-636 inhibitory potency at GluN1/2A receptors at hyperpolarized membrane potentials is caused primarily by effects on the association rate of ArgTX-636 to the NMDAR ion channel; an effect that also has been observed for memantine⁴⁶. Interestingly, similar studies of the voltage-dependency of binding kinetics for channel blockers such as ketamine and MK-801 have suggested an opposite pattern where voltage-dependence of NMDAR block mainly was controlled by effects of membrane potential on compound dissociation rates¹⁰.

Trapping of ArgTX-636 in the NMDAR channel

NMDAR channel blockers can be classified according to their ability to allow the ion channel to close while the channel blocker is bound. Compounds that allow the channel to close around the bound blocker are denoted trapping blockers as they cannot escape from the channel once the receptor has deactivated^{8; 10; 52; 57; 58; 59}. The blocking mechanism of ArgTX-636 inhibition of iGluRs has only been described for AMPARs, where ArgTX-636 has been shown to be a trapping blocker⁶⁰. To determine whether ArgTX-636 is a trapping blocker of the GluN1/2A ion channel, we used the recording protocol described in Fig. 3. First, whole-cell GluN1/2A currents were evoked by rapid application of 100 μ M Glu followed by rapid shifting of the cell into 500 nM ArgTX-636 in the continued presence of agonist for 6 seconds to produce a steady-state block of $96 \pm 1\%$ of the current (Fig. 3A). Next, Glu and ArgTX-636 were simultaneously removed by rapidly shifting the cell into bath solution (Fig. 3); hereby rapidly removing the agonist followed by receptor deactivation and potential trapping of ArgTX-636 in the closed-channel state. The cell was then held in the bath solution for a time period (t) until rapid application of 100 μ M Glu. As shown in Fig. 3B for a representative cell, the second application of Glu evoked a two-component current-response (I_{recovery}). The initial component describes the rapid activation (10-90% rise time: <5 ms) of the fraction of receptors that are not blocked by ArgTX-636, whereas the current increase during the second component is much slower (10-90% rise time: >10 s) and describes unbinding of ArgTX-636 that have remained in the channel during t (Fig. 3B). Measurement of the peak amplitude of the fast component of I_{recovery} at increasing t were then used to determine whether ArgTX-636 can escape or unbind from deactivated NMDARs (Fig. 3C). The results showed that I_{recovery} did not increase from the current at steady-state open-channel block and remained constant with increasing t (Fig. 3D). These data conclusively demonstrate that ArgTX-636 acts as a trapping blocker of GluN1/2A

receptors, and suggest that ArgTX-636 has a mechanism-of-block at NMDARs that is similar to its previously determined mechanism at AMPARs⁶⁰. Detailed analysis of ArgTX-636 block of kainate receptors is currently not available. However, the mechanism of block for the philanthotoxin analogue PhTX-343 has been investigated at recombinant kainate receptors using fast application protocols similar to the present study⁶¹. Here it was found that PhTX-343 acts as a use- and voltage dependent trapping blocker; suggesting that polyamine toxins overall have similar mechanism of block at all iGluR subtypes although it should be noted that ArgTX-636 is larger and has a different aromatic head group than PhTX-343. A major difference, however, was the observation that onset of PhTX-343 block of kainate receptors followed a double-exponential time course and that the apparent K_d was independent of membrane potential at hyperpolarized potentials; likely indicating that PhTX-343 can permeate the channel at negative membrane potentials. Whether these properties extend to the larger ArgTX-636 awaits experimental investigation.

We also investigated if the trapped ArgTX-636 can act as a so-called ‘foot-in-the-door’ blocker; i.e. prevent dissociation of the agonist from LBDs by locking the receptor conformation to prevent the clam-shell shaped LBD to undergo conformational changes that are needed to release the agonist. Briefly, comparison of measured receptor time constants for deactivation in the absence and presence of ArgTX-636 showed no significant difference, which indicate that bound ArgTX-636 does not prevent receptor deactivation and agonist unbinding (Supplemental figure S1). In summary, our electrophysiological analysis of ArgTX-636 block of recombinant GluN1/2A receptors demonstrate that ArgTX-636 is a trapping blocker; i.e. that the molecule can be stably accommodated within the channel in the closed state.

Effect of alanine and glycine mutagenesis in the M2 -loop segment on ArgTX-636 block of NMDARs

Previous work on the molecular binding mode of ArgTX-636 include computational docking into models of the GluN1/2A ion channel based on the K⁺ channel X-ray crystal structures in combination with structure-activity relationship analysis of ArgTX-636 analogs^{24; 49}. These studies propose that ArgTX-636 is positioned deep in the outer vestibule with the aromatic head group of the toxin located at the selectivity filter and the polyamine tail protruding into the channel pore (Fig. 4). The selectivity filter is formed by a ring of Asn residues (N616 in GluN1; N614 in GluN2A; both denoted the N position) located at the apex between the ascending M2-helix and the descending M2-pore loop that forms the ion channel pore (Fig. 4). The M2-loop is thought to adopt an extended or random coil structure with consecutive amino acid residues exposed to the lumen of the channel^{5; 32}(Fig. 4). To elucidate the contribution of the side chains of individual residues in the selectivity filter and the M2-loop for ArgTX-636 binding, we performed Ala and Gly scanning mutagenesis of all amino acid positions between the selectivity filter and the C-terminal end of the M2-loop (Glu621 in position N+5 in GluN1 and Asn621 in position N+7 in GluN2A)(Fig. 4, 5 and 6).

Using two-electrode voltage-clamp electrophysiology to measure agonist-evoked steady-state currents from wild-type (WT) and mutant GluN1/2A receptors expressed in *Xenopus*

oocytes (*Materials & Methods*), we determined the impact of Ala and Gly substitutions on ArgTX-636 binding by determining the percentage steady-state block produced by 100 nM ArgTX-636 at a holding potential of -60 mV. This concentration was chosen since 100 nM ArgTX-636 inhibits WT GluN1/2A approximately 50% at a membrane potential of -60 mV (Fig. 5 and 6). We assume that the observed differences in steady-state inhibition by a single concentration of ArgTX-636 at a given mutant compared to steady-state inhibition at WT GluN1/2A primarily reflects changes in binding affinity of the toxin. However, we did not investigate potential effects of mutations on gating properties and this interpretation is therefore dependent on the idea that the mutations do not profoundly change receptor properties such as channel open probability. Because we were interested in the pattern of changes in ArgTX-636 block produced by scanning mutagenesis of the M2-pore loop segment, we did not determine IC_{50} of ArgTX-636 at each mutant and therefore cannot report the quantitative effect of the individual mutations on ArgTX-636 binding. We also included measurements of steady-state inhibition by the prototypical small-molecule NMDAR channel blocker MK-801 as well as Mg^{2+} . These two blockers are well-studied NMDAR channel blockers, and thus were included as reference compounds.

The results of the Ala and Gly scanning mutagenesis of GluN1 and GluN2A M2-loop residues, respectively, are summarized in Fig. 5 and Fig. 6. For GluN1, we found that Gly substitution, which removes the WT residue side-chain, produced significant changes in ArgTX-636 block at all positions from N616 (that forms the selectivity filter) to E621 at the predicted intracellular end of the GluN1 M2-loop. Note that in WT GluN1 positions 618 and 620 already contain Gly (Fig. 5B). At S617, I619 and E621, block was reduced substantially to less than 15% by Gly substitution, whereas removal of the Asn side chain at N616 in the predicted selectivity filter increased block to 78%. This pattern for ArgTX-636 was in contrast to the patterns observed for block by 50 nM MK-801, for which only Gly substitution at N616 and S617 in GluN1 substantially decreased block from 75% at WT GluN1/2A to less than 40% (Fig. 5B). Block by 0.5 mM Mg^{2+} was significantly decreased by Gly substitutions at all M2-loop positions; however only substantially upon substitutions at N616 and I619; from 88% at WT GluN1/2A to 28% and 53% at GluN1-N616G/2A and GluN1-I619G/2A, respectively (Fig. 5B). The Ala scanning of the GluN1 M2-loop residues showed significant and substantial reduction in ArgTX-636 block at all residues except S617 and G618A; from 47% block at WT GluN1/2A to less than 20% (Fig. 5C). In contrast, ion channel block by MK-801 and Mg^{2+} was mostly affected by Ala substitutions at residues in or close to the apex of the M2-loop of GluN1 (Fig. 5C).

For the Ala and Gly scanning mutagenesis of GluN2A, we found that Gly substitution of M2-loop residues increased block by 100 nM ArgTX-636 from 48% at WT GluN1/2A to almost 100% at position N614, N615, S616, V617 (Fig. 6). Gly substitutions at P618, V619 and Q620 did not significantly change block (Fig. 6B). For MK-801 and Mg^{2+} , substitutions at N614 to P618 generally reduced block (Fig. 6B). Introduction of Ala in the M2-loop of the GluN2A subunit generally followed the same qualitative pattern as the Gly scan on ArgTX-636, MK-801 and Mg^{2+} block (Fig. 6C). ArgTX-636 block increased upon Ala substitutions at positions close to the M2-loop apex (N614, N615 and S616) whereas MK-801 and Mg^{2+} block decreased. In contrast, Ala substitutions at the C-terminal positions

in the M2-loop (V617 to N621) generally produced smaller effect on the ion channel blockers (Fig. 6C).

Role of M2-loop residues for ArgTX-636, MK-801 and Mg^{2+} block of the GluN1/2A ion channel

The results of the Gly and Ala mutational scanning of the GluN1 and GluN2A M2-pore loops are summarized in Fig. 7, which provides a functional map defining the role of pore-lining residues for block by ArgTX-636, MK-801 and Mg^{2+} . For GluN1, it can be seen that ArgTX-636 block is sensitive to mutations along the entire M2-loop (*N* to *N*+5 positions), whereas MK-801 is sensitive to mutations only at the apex of the M2-loop (*N* and *N*+1 positions) and Mg^{2+} block is affected by mutations in the *N* and *N*+3 positions. These results suggest that ArgTX-636 is in contact or in close vicinity with the entire GluN1 M2-loop when bound in the GluN1/2A ion channel. These data are consistent with the proposed binding mode for ArgTX-636 in which the polyamine tail protrudes through the core of the ion channel and interacts directly with N616, I619 and E621 in GluN1 (*N*, *N*+3 and *N*+5 positions, respectively)(Fig. 4). The observation that MK-801 and Mg^{2+} block is decreased by Ala and Gly substitutions in the *N* and *N*+1 positions in GluN1 are consistent with previous extensive studies of the role of these residues for binding of these two channel blockers ^{27; 28; 33; 35; 40; 41}.

For GluN2A, the mutational scans of the M2-loop positions showed overall similar patterns of sensitivity for ArgTX-636, MK-801 and Mg^{2+} , as mutations of the *N* to *N*+4 positions generally affected all three blockers whereas mutation in the *N*+5 and *N*+6 positions only induced smaller effects. As mentioned previously, decrease in MK-801 and Mg^{2+} block observed upon removal of the hydrophilic Asn residues in the *N* and *N*+1 positions are consistent with the well-established idea that these residues together with the equivalent Asn residue in GluN1 are key interaction partners for MK-801 and Mg^{2+} as well as other small-molecule channel blockers. Interestingly, mutations of the *N* to *N*+4 positions increased ArgTX-636 block. Within the framework of the ArgTX-636 binding model (Fig. 4), these results are surprising because the proposed hydrogen bond interactions between ArgTX-636 and the side chains of Asn and Ser in the *N* and *N*+2 positions, respectively, are perturbed by Ala and Gly substitutions and were thus expected to result in decreased ArgTX-636 block. We decided to further explore this inconsistency of the binding model relative to residues in and around the selectivity filter by substitution of the *N* position in GluN1 with Arg and the *N* and *N*+1 position in GluN2A with Arg, Ser and Cys. Following the same protocol described for the Ala and Gly scanning mutagenesis, we determined how these mutations affected ion channel block of 100 nM ArgTX-636 (Supplemental figure S2). Insertion of an Arg residue in the *N* position of GluN1 decreased ArgTX-636 block, which is consistent with the binding model by potentially introducing steric clash in to the selectivity filter (Fig. 4). For GluN2A, mutation of Asn in *N* and *N*+1 to Arg also disrupted ArgTX-636 block, whereas mutation to smaller and polar residues (Ser and Cys) increased block (Supplemental figure S2); although not to the same degree as Gly and Ala. These data show that decreasing the bulk size of the amino acid side chain in position *N* and *N*+1 of GluN2 improve ArgTX-636 binding; suggesting that Asn side chains in these positions are not involved in direct hydrogen bond interactions with ArgTX-636. According to the ArgTX-636 binding

model the polyamine tail is binding in close proximity of the *N*+5 and *N*+6 positions (P618 and V619, respectively) of the GluN2A subunit. Specifically, it has been suggested that the terminal guanidinium group of the toxin forms a direct interaction with the backbone carbonyl of V619 (Fig. 4). Although substitution of V619 for Ala or Gly only induced modest effects on ArgTX-636 block (Fig. 5), this hypothesis cannot be further examined, as backbone interactions in this highly conserved domain are unlikely to be affected by our mutations.

Role of the *N*+3 position in GluN1 and GluN2A for Mg²⁺ block

In addition to direct effects on protein-ligand interactions, single-point mutations in the M2-loop might also affect ligand-binding by long-range allosteric effects on the overall channel conformation. In this regard, it is noteworthy that mutation of the *N*+3 position in both the GluN1 and GluN2A subunit (Ile619 and Val617, respectively) greatly reduced channel block by Mg²⁺ (Fig. 7). In accordance with our Ala and Gly scanning mutagenesis, early studies have shown that mutation of the Asn residues in the selectivity filter of GluN1 and GluN2 subunits influence Mg²⁺ block, indicating that these residues may form part of the binding site for the divalent cation. In contrast, the *N*+3 position in both GluN1 and GluN2A is located in the middle of the M2-pore loop segment, and according to homology models of the GluN1/2A ion channel, the side chains of the hydrophobic Ile and Val residues in the *N*+3 positions are pointing away from the channel (Fig. 4 and 5). Taken together, it seems unlikely that the *N*+3 residues are interacting directly with Mg²⁺. Interestingly, in the homology model the *N*+3 residues in the M2-pore loop are located in close proximity of conserved Trp residues on the M2-helix (W608 in GluN1 and W606 in GluN2A, respectively) (Supplemental Fig. S3), that previously have been shown to hold an important role for channel block by Mg²⁺ in NMDARs (Williams et al. 1998; Retchless et al. 2012). Hence, it seems likely that Ala and Gly substitutions of the *N*+3 residues will affect hydrophobic residue-residue interactions to the Trp residues on the M2 helix, and thereby allosterically reduce ion channel block by Mg²⁺.

Differential contribution of the M2-loops of GluN1 and GluN2A to binding of channel blockers

Structures of homotetrameric ion channels such as K⁺ channels show identical channel-lining elements from each subunit to be arranged with near perfect fourfold rotational symmetry around the channel center axis, such that the M2 and M3 segments are aligned vertically and horizontally and contribute equally to the channel interior⁶². However, NMDARS are obligatory heteromers and thus cannot have fourfold rotational symmetrical arrangement of the M2 and M3 segments from GluN1 and GluN2, which present a caveat for models of the NMDAR channel built from K⁺ channels structures. Furthermore, the M2 and M3 segments from GluN1 and GluN2A may not be vertically aligned with each other^{37; 38; 42; 63}. Studies using substituted cysteine accessibility mutagenesis (SCAM) to probe external accessibility of residues in the NMDAR ion channel has suggested that the M3 segment from GluN1 and GluN2 subunits are staggered relative to each other in the vertical axis of the channel such that GluN2 positions in M3 is located more external than homologous positions in GluN1⁴². Whether such differences in vertical alignment also apply to the M2-loop is unknown. However, it is noteworthy that we find mutations across

all *N* to *N*+5 positions in the M2-loop of GluN1 to perturb ArgTX-636 block whereas mutations of only the *N* to *N*+3 positions in GluN2A M2-loop perturb block (Fig. 7). This may suggest that the pore-lining part of the M2-loop in GluN1 and GluN2A are not vertically aligned. Further mutational analysis of the M2-loop paired with structure-activity relationship (SAR) studies of ArgTX-636 analogs with modifications in the polyamine tail may seem warranted as a potential method to probe the organization of GluN1 and GluN2 M2-loops relative to each other.

In summary, we have analyzed the time-course for binding and unbinding of ArgTX-636 as well as the molecular mechanism of ArgTX-636 block at recombinant GluN1/2A receptors. We found that ArgTX-636 is a trapping blocker with strong voltage-dependency compared to other classes of NMDAR channel blockers. Mutational analysis of the M2-loops in GluN1 and GluN2A was performed to evaluate a previously proposed model for ArgTX-636 interaction with the NMDAR pore region of the channel. Together, our results advance the molecular and kinetic understanding of ArgTX-636 channel block of NMDARs, which is of use for future rational design of argiotoxin-based blockers with tailor-made properties.

Materials and Methods

Reagents

Unless otherwise noted, all chemicals and ligands were purchased from Sigma (St. Louis, MO). Cell-culturing reagents were from Invitrogen (Carlsbad, CA). ArgTX-636 was synthesized as previously described ²⁴.

DNA constructs and mutagenesis

Rat cDNAs for GluN1-1a (GenBank accession number U08261; hereafter, GluN1) and GluN2A (D13211) were provided by Drs. S. Heinemann (Salk Institute) and S. Nakanishi (Osaka Bioscience Institute). We use a numbering nomenclature for GluN1 and GluN2A amino acid positions in which residues are reported relative to amino acid position 1 in the coding sequence of GluN1 and GluN2A. GluN1 and GluN2 mutants were constructed by the Quikchange site-directed mutagenesis method (Stratagene). All mutagenesis reactions were performed using *Pfu* polymerase (Stratagene), and the resulting mutant constructs were sequence verified.

HEK cell transfection and patch-clamp electrophysiology

HEK293 cells (Clontech, Mountain View, CA) were plated in 24-well plates on glass coverslips coated with 0.1 mg/ml poly-D-lysine approximately 48 hours prior to the experiments. The culture medium was Dulbecco's Modified Eagle Medium with GlutaMax-I (Invitrogen, Carlsbad, CA) supplemented with 10% dialyzed fetal bovine serum, 10 U/ml penicillin, and 10 µg/ml streptomycin. Cells were transfected with 0.5 µg/well plasmid cDNAs encoding EGFP, GluN1 and GluN2 subunits at a ratio of 1:1:1 using the calcium phosphate precipitation method as previously described ⁶⁴. In order to minimize cytotoxicity, the culture medium was replaced 4-5 hours following addition of the transfection mix with medium containing NMDAR antagonists D,L-2-amino-5-

phosphonovalerate (200 μ M) and 7-chlorokynurenic acid (200 μ M). Experiments were performed approximately 24 hours following transfection.

Whole-cell voltage-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) at room temperature (23°C). Recording electrodes (3–4 M Ω) were made from thin-walled glass micropipettes (TW150F-4; World Precision Instruments, Sarasota, FL) pulled using a horizontal puller (P-1000; Sutter Instrument Company, Novato, CA). The electrodes were filled with internal solution composed of (in mM) 110 D-gluconate, 110 CsOH, 30 CsCl, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA, 2 NaATP, and 0.3 NaGTP (pH 7.35 with CsOH). The extracellular recording solution was composed of (in mM) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl₂, 0.01 EDTA (pH 7.4 with NaOH) and supplemented with 20 mM D-mannitol. Rapid solution exchange was achieved using a two-barrel theta-glass pipette controlled by a piezoelectric translator (Burleigh Instruments, Fishers, NY). Junction currents between undiluted and diluted (1:2 in water) extracellular recording solution were used to estimate speed of open tip solution exchange after recordings and typically had 10–90% rise times of 0.5–1.5 milliseconds (*data not shown*). All ligand stock solutions were prepared in extracellular recording solution.

Two-Electrode Voltage-Clamp Recordings

Preparation and cRNA injection (1 ng GluN1 and 10 ng GluN2A per oocyte) of *Xenopus* oocytes were performed essentially as previously described⁵⁴. Recordings were performed using the following recording buffer (in mM): 115 NaCl, 2 KCl, 5 HEPES, and 1.8 BaCl₂; pH 7.6.

Data analysis

Macroscopic response time-courses of NMDAR responses were analyzed using Clampfit software (Molecular Devices, Union City, CA) and plotted using Origin software (Microcal Software, Northampton, MA). The macroscopic blocking time constant (τ_{block}) and macroscopic unblocking time constant (τ_{unblock}) were measured by fitting the time course of current block and recovery with a single exponential function:

$$I(t) = I_{ss} + (I_0 - I_{ss}) \exp(-t/\tau),$$

where $I(t)$ is the amplitude of the current at time t , I_{ss} is the current at steady state, I_0 is the current at time zero, τ is the time constant of decay. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Unless otherwise noted, results are expressed as mean $\pm$ SEM. Statistical analysis of pairwise or multiple comparisons were performed using ANOVA, or student's t-test as appropriate. $p < 0.05$ was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The NMDA receptor is an important ligand-gated ion channel in the brain.
- Small-molecule ligands that bind and block the NMDA receptor ion channel have therapeutic potential for treatment of brain diseases.
- We examine the mechanism of block for the spider polyamine toxin Argiotoxin-636 at the GluN1/2A-type NMDA receptor.
- Argiotoxin-636 is a fast binding and unbinding trapping blocker of GluN1/2A receptor.
- Mutational analysis of M2-loop segments in GluN1/2A identify residues important for Argiotoxin-636 block.

Figure 1. NMDAR structure. *A.* Homology model of the GluN1/2A receptor (*left*) and a single GluN1 subunit (*right*). GluN1 and GluN2 subunits share a highly similar modular structure that contains an N-terminal domain (NTD), a ligand-binding domain (LBD) that are connected by flexible linkers to a transmembrane domain (TMD) consisting of three alpha-helical membrane-spanning segments (M1, M3, and M4) and a short membrane re-entrant segment (M2) containing a short N-terminal alpha helix and a C-terminal loop. Each of the four LBD contains a single agonist binding site with specificity for Gly in GluN1 and Glu in GluN2 subunits. Omitted from the model are intracellular C-terminal domains that expand from the M4 segment in each subunit and contains multiple sites involved protein-protein interactions with synaptic scaffolding proteins and signaling enzymes. The GluN1/2A model has previously been described by Acker *et al*⁶⁵. *B.* Chemical structure of ArgTX-636. *C.* The core of the ion channel of GluN1/2A is formed by the M2 and M3 segments from the GluN1 (*green*) and GluN2 (*grey*) subunits. The M2 segment contains an alpha-helical part and a loop that lines the pore. A ring of Asn residues at the apex of the M2 helix and the M2-loop constitute the narrowest part of the channel. M2-loop positions in GluN1 and GluN2A subjected to mutational analysis in the present work are high-lighted with their α -carbon in orange. The shape and dimensions of the channel inner lining are visualized as gray surface

contours. The model of the GluN1/2A ion channel has previously been described in Nelson *et al*²⁴.

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Figure 2.

Time-course of ArgTX-636 open-channel block of GluN1/2A receptors. *A.* Whole-cell currents recorded from HEK293 cell expressing GluN1/2A receptors held at -80 mV in the continued presence of $50 \mu\text{M}$ Gly with application of $100 \mu\text{M}$ Glu and ArgTX-636 (500 nM) in the indicated periods. The rates for the onset ($1/\tau_{\text{block}}$) and offset ($1/\tau_{\text{unblock}}$) of open-channel block are calculated by fitting an exponential function (*Materials & Methods*) to the phases of current decay and recovery (indicated by gray stipulated boxes). *B.* The rates for onset ($1/\tau_{\text{block}}$) and offset ($1/\tau_{\text{unblock}}$) of open-channel block plotted versus ArgTX-636 concentration. The rate of block is dependent on the concentration of blocker, whereas the rate for unblock is independent of blocker concentration. The association and dissociation rate constants were calculated by linear regression analysis of the $1/\tau_{\text{block}}$ data points using the function $1/\tau_{\text{block}} = K_{\text{off}} + [\text{ArgTX-636}] K_{\text{on}}$, where K_{off} and K_{on} are the dissociation and association rate constants, respectively, and $[\text{ArgTX-636}]$ is the concentration of ArgTX-636. Data are mean $\pm$ SEM from 3 to 11 cells for each ArgTX-636 concentration. *C.* Voltage-dependency of block. Representative current traces are shown from the same cell subjected to the same application protocol as in A, but at a range of membrane potentials from -40 to -100 mV. *D.* Observed rates are shown for open-channel block and unblock at 500 nM ArgTX-636 as function of membrane potential (V_m). Both the rates of onset and offset of block are dependent on membrane potential. Data are mean $\pm$ SEM from 3 to 7 cells for each holding potential.

Figure 3.

Trapping of ArgTX-636 in the GluN1/2A channel after blockade. *A.* Representative current trace obtained from cell expressing GluN1/2A receptors held at -80 mV in the presence of 50 μ M Gly. The cell is first subjected to rapid application of a saturating concentration of Glu (100 μ M) for 6 s to produce maximum receptor activation ($I_{control}$). Application of 500 nM ArgTX-636 is then performed for 6 s to produce near-complete steady-state channel block (I_{block}). Trapping of ArgTX-636 was assessed by rapidly moving the cell from ArgTX-636 + Glu to bath solution for a given time period (Δt ; indicated with arrow heads) before rapid reapplication of Glu was performed to evoke a response ($I_{recovery}$) that reflects receptors not blocked by ArgTX-636 and subsequently slower recovery from block by dissociation of ArgTX-636. *B.* Expanded view of the initial phase of $I_{recovery}$ from the current trace in A (indicated by stipulated box). The initial fast component of the current response represent activation of the fraction of receptors not blocked by ArgTX-636 (indicated by dotted grey line) and the subsequent more slow component represent unbinding of ArgTX-636. *C.* Overlay of different current traces from the same cell subjected to the stimulation protocol described in A, but with increasing Δt periods. *D.* Plot of recovery as function of increasing periods of time spent in wash solution between steady-state receptor block and re-application of Glu. For comparison the time-course of current recovery from ArgTX-636 block in the continued presence of agonist is shown. Data represent the mean + SEM from 3 to 11 cells. The data indicate that recovery from ArgTX-636 block only is achieved in the presence of agonist; showing that the toxin molecule can be trapped in the closed NMDAR channel.

Figure 4. Model of the binding mode of ArgTX-636 in the GluN1/2A ion channel. The M3 and M2 segments of GluN1 (*green*) and GluN2A (*grey*) are shown as cartoon representations with ArgTX-636 (*yellow*) and M2-loop residue side chains displayed as sticks (*orange*). Predicted interactions between ArgTX-636 and M2-loop residues are indicated with stipulated lines. Model is taken from ²⁴.

Figure 5.

Ala- and Gly-scanning mutagenesis of the M2-pore loop in GluN1 disrupts channel block by ArgTX-636, MK-801 and Mg²⁺ with distinct patterns of sensitive and insensitive residues. *A.* Overview of the M2 pore loop residues in GluN1 that were subjected to Gly and/or Ala substitution. *B and C.* Graphical summary of percent inhibition by 100 nM ArgTX-636 (*left*), 50 nM MK-801 (*middle*), and 0.5 mM Mg²⁺ (*right*) at WT and GluN1-substituted mutant GluN1/2A receptors expressed in *Xenopus* oocytes. Data represent mean + SEM for experiments at 4 to 8 oocytes. * $P < 0.05$ compared to WT GluN1/2A (ANOVA).

Figure 6. Ala- and Gly-scanning mutagenesis of the M2 pore loop in GluN2A disrupts channel block by ArgTX-636, MK-801 and Mg²⁺ with distinct patterns of sensitive and insensitive residues. *A*. Overview of the M2 pore loop residues in GluN2A that were subjected to glycine and/or alanine substitution. *B* and *C*. Graphical summary of percent inhibition by 100 nM ArgTX-636 (*left*), 50 nM MK-801 (*middle*), and 0.5 mM Mg²⁺ (*right*) at WT and GluN2A-substituted mutant GluN1/2A receptors expressed in *Xenopus* oocytes. Data represent mean + SEM for experiments at 4 to 8 oocytes. * $P < 0.05$ compared to WT GluN1/2A (ANOVA).

Figure 7.

Functional map of the impact of Ala and Gly mutations on ion channel block. Shown is the M2 pore-helix and pore loop of GluN1 (*green*) and GluN2A (*grey*) with positions in the M2 pore-loop subjected to mutation indicated by spheres numbered according to their position relative to the Asn that form the entrance to the pore. Red spheres indicate loss of channel block while blue spheres indicate gain of channel block by mutation to Ala and/or Gly (>30% difference in channel block compared to WT GluN1/2A). The numbering of residue positions used (*N*, *N+1*, *N+2*, etc.) indicates the position relative to the Asn residue in apex of the M2-loops of GluN1 (N616) and GluN2A (N614) that forms the pore selectivity filter.