

Modeling of Glutamate GluR6 Receptor and Its Interactions with Novel Noncompetitive Antagonists

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The study proposes the first complete model of an ionotropic glutamate receptor (GluR6). The model is in accordance with available experimental data from single-particle electron microscopy images and exhibits correct shape and dimensions and the appropriate symmetry: 2-fold in the N-terminal domain (NTD), ligand-binding domain (LBD), and external part of the transmembrane region, whereas it is 4-fold deeper in the channel. The methodology applied for GluR6 receptor model building was validated in the docking procedure of competitive and uncompetitive antagonists. The constructed model was used to study molecular interactions of novel noncompetitive GluR6 antagonists with their molecular target. A new binding site in the GluR6 receptor transduction domain has been identified. It is situated between two subunits in the receptor dimer. The following residues were recognized as crucial for interactions: Arg663A, Arg663B (M3-S2 linker), Ser809B (S2-M4 linker), and Phe553A (S1-M1 linker).

INTRODUCTION

L-Glutamate mediates most of the excitatory neurotransmission in the mammalian central nervous system by activation of two main families of glutamate receptors, namely ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Based on their structural similarities and pharmacological properties, three different groups of mammalian iGluRs have been identified: N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) subtypes, and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainic acid, KA) receptors.

The glutamatergic system, including ionotropic glutamate receptors, remains an attractive molecular target for pharmaceutical intervention.^{1,2} Glutamate excitotoxicity has been implicated in the pathogenesis of many neurological diseases, such as stroke, amyotrophic lateral sclerosis, and epilepsy³ and chronic neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, and Huntington's chorea.^{4,5} Thus, especially antagonists of iGluRs are important and promising medicinal substances.

Alzheimer's and Parkinson's diseases are listed among the most serious health problems in the industrialized world. It is estimated that about 15–18% of the population over the age of 65 suffer from different types of dementia, including Alzheimer's disease. Parkinson's disease afflicts over 1.5% of population over the age of 65. The glutamatergic system is one of the targets for novel drugs for Alzheimer's and Parkinson's diseases. The registered drug for Alzheimer's

disease treatment, memantine, is an uncompetitive blocker of NMDA receptors.

The knowledge of the function and physiological importance of proteins is the key requirement for the design of novel, biologically active compounds with potential pharmaceutical, industrial, and agricultural applications. The function of a protein is clearly combined with its three-dimensional structure. When experimental data are unavailable, molecular modeling, especially based on homology, is a useful method for obtaining coordinates of proteins of unknown structures. Although homology modeling has been successfully applied to a great number of proteins, including G protein-coupled receptors, modeling of ligand-gated ion channels, in particular iGluRs, remains a challenge,^{6,7} primarily because of the lack of suitable templates and second - because of the difficulties which emerge at joining together particular domains of the receptor. Moreover, a significant problem is the symmetry of iGluR tetramers.

Ionotropic glutamate receptors are homomeric or heteromeric tetramers. Each subunit comprises N-terminal domain (NTD), ligand binding domain (LBD), transmembrane channel region, and C-end (compare Figure 1). All three main domains (apart from C-end) have been modeled. Dimers of NTD were constructed by De Luca et al. for AMPA receptor⁸ and by Huggins and Grant⁹ and Marinelli et al.¹⁰ for NMDA receptors. In these studies extracellular fragments of the solved mGluR or LIVBP (leucine-isoleucine-valine-binding periplasmic bacterial protein) were applied as templates. The models of LBD of ionotropic glutamate receptors were built applying another periplasmic bacterial protein (lysine-arginine-ornithine binding protein, LAOBP) as templates. Compared with X-ray structures of LBD, they turned out to be a good approximation of this glutamate receptor fragment,¹¹ in spite of the low sequence identity of about 12%. The

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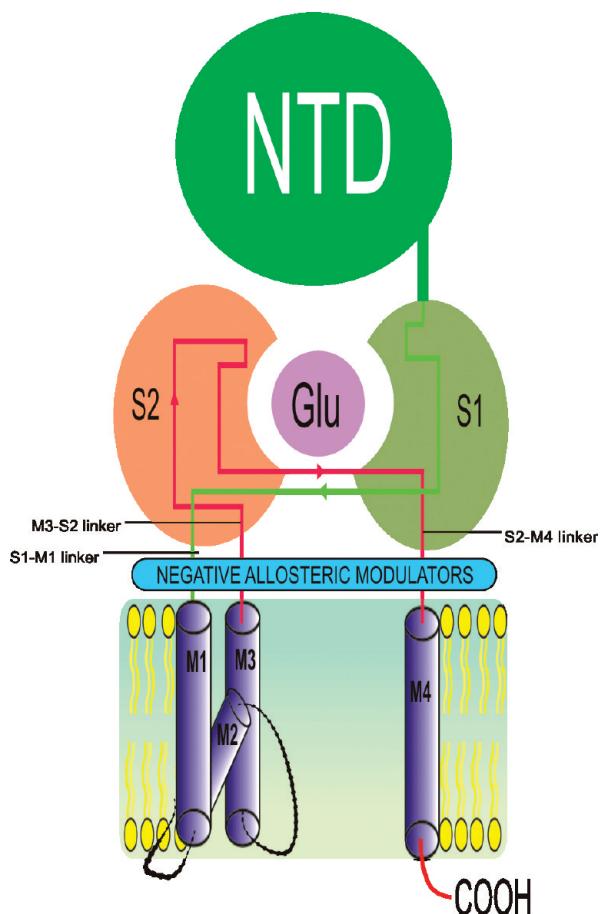


Figure 1. The scheme of an ionotropic glutamate receptor.

transmembrane region of the NMDA receptor was modeled by Bachurin et al. (closed-state model)¹² and Tikhonova et al.¹³ (open-state model). However, apart from the early models based on pentameric topology,¹⁴ no models of complete ionotropic glutamate receptors are available.

Recently, we have constructed¹⁵ the first model of the glutamate receptor ion channel (GluR5) which included the M4 helix and exhibited the correct 2-fold rotational symmetry as indicated by experimental data. Moreover, we have built the first model of the GluR5 receptor transduction domain to connect the ion channel with the LBD tetramer. A few months later, Speranskiy and Kurnikova¹⁶ proposed a model of GluR2 receptor S1-M1 and M3-S2 linkers. In this paper we present the first complete closed-form model of glutamatergic ionotropic receptor (GluR6), which possesses N-terminal domain, ligand-binding domain, and transmembrane region connected via necessary linkers. The methodology applied in the model building was validated in the procedure of docking of competitive and uncompetitive antagonists.

The obtained model was used to investigate interactions of novel noncompetitive GluR6 receptor antagonists (which we have recently synthesized¹⁷) with their molecular target. The problem of binding site of kainate noncompetitive antagonists remains an open question as there are a few known compounds of this type **1–3**^{18–20} (compare Figure 2) and no binding site was proposed for them. However, experimental studies for AMPA receptors by Balannik et al.²¹ were used as a starting point for docking procedure.

Thus, the aim of the presented work was the construction of the GluR6 kainate receptor model in the closed form,

validation of the applied methodology by docking of competitive and uncompetitive GluR6 antagonists, and identification of the novel binding site for noncompetitive antagonists in the docking procedure.

MATERIALS AND METHODS

1. GluR6 Receptor Model Building. Each domain of the GluR6 receptor (N-terminal domain, ligand-binding domain, transmembrane region) was modeled separately. In all the cases the main technique was homology modeling. Moreover, to model NTD and LBD domain tetramers, protein–protein docking was applied.

Modeling of the transmembrane region of GluR6 was started with the prediction of a secondary structure with public-accessible servers: PredictProtein²² and Jpred²³ as well as transmembrane regions with DAS Server²⁴ and TMHMM Server v. 2.0.²⁵ To model the M4 transmembrane segment, the HHpred server²⁶ was applied for identification of potential templates. Among several possible templates protein PET L (PDB code 1VF5),²⁷ MERF membrane protein (PDB code 2H3O),²⁸ a hypothetical protein in the COX14-COS3 intergenic region precursor (PDB code 1RP4),²⁹ and a major coat protein of PF1 virus (PDB code 1PFI)³⁰ were selected. Multiple alignment was performed with MAFFT³¹ by the accurate and slow method at default parameters. In all the cases the alignment was refined manually.

Modeller9v1³² was applied for homology modeling. For Modeller9v1³² calculations were performed on the graphical station HP xw 4400, Intel CoreDuo 2 6300, 1.86 GHz, 2GB RAM, Windows XP Professional. Protein–protein docking was carried out with the application of the SymmDock server.³³

Linkers, constituting the transduction domain, were modeled with the application of several techniques and tools (ab initio Rosetta methodology,³⁴ Modeller9v1³² dope-loop model, and Biopolymer ModelLoop incorporated in Sybyl7.3). Sybyl7.3 calculations were carried out on the graphical station 2xXeon2000, 3 GHz, 1GB RAM, Fedora Core 4. Finally, the GluR6 receptor model was refined with the application of Yasara Dynamics.³⁵ The quality of the final model was verified with VERIFY3D,³⁶ SOLVX,³⁷ ANOLEA,³⁸ and WHAT IF³⁹ server.

2. Ligand Preparation, Binding Site Identification, and Ligand Docking. The energy and geometry of the ligands were first optimized with the ab initio method in Hartree–Fock approximation with the application of the 6-31G* basis set of Spartan06, next the obtained structure was subjected to conformational analysis with a GA Conformational Search of Sybyl7.3 (with simulation of water as a solvent), and, finally, the lowest-energy conformer was optimized as in the first step. Spartan06 calculations were performed on the graphical station HP xw 4400, Intel CoreDuo 2 6300, 1.86 GHz, 2GB RAM, Windows XP Professional. The GA Conformational Search of Sybyl7.3 was selected for conformational analysis as it allows for obtaining good results in a relatively short time.⁴⁰

The binding site for novel GluR6 noncompetitive antagonists was identified based on experimental results by Balannik et al.²¹ for AMPA receptor noncompetitive inhibitors.

Docking was performed with the flexible docking method of Surflex⁴¹ incorporated in Sybyl7.3. Surflex⁴¹ is a fully

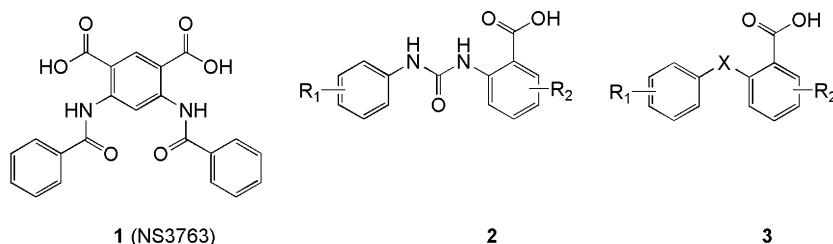


Figure 2. Noncompetitive antagonists of kainate receptors.

automatic flexible molecular docking algorithm that combines the scoring function from the Hammerhead docking system with a search engine that relies on a surface-based molecular similarity method as a means to rapidly generate suitable putative poses for molecular fragments.⁴¹ The side chain conformations of residues constituting the binding pocket in an obtained ligand–receptor complex were optimized with Yasara Dynamics³⁵ with the application of the Yamber3 force field. This allowed for optimizing the conformations of the residues constituting the binding pocket and for gaining the final docking results (see the Supporting Information for the tables with results).

PyMol,⁴² Vega,⁴³ Chimera,⁴⁴ SPDBV,⁴⁵ and Yasara³⁵ were used for visualization of results. All graphics were produced with PyMol⁴² and Chimera.⁴⁴

RESULTS

1. GluR6 Model Building. Each domain of the GluR6 receptor (Swiss Protein Data Bank accession number Q13002) was modeled separately. The methodology of construction of the GluR6 receptor LBD tetramer, the transmembrane channel region, and the transduction domain was analogous to the one previously described for the GluR5 receptor.¹⁵

Thus, the first step of the GluR6 receptor model construction was building a model of a tetramer of the GluR6 ligand binding domain. Sequence alignment was performed with MAFFT³¹ by the accurate and slow method and refined manually. The sequence identity between the target and the AMPA GluR2 receptor LBD complexed with an antagonist DNQX (PDB code 1FTL⁴⁶) selected as a template was 53.39%. The available crystallographic structures for GluR6 LBD have not been used, as there are no solved structures of GluR6 LBD containing an antagonist. Moreover, the selected template was characterized by the favorable relative orientation of two subunits in a considered dimer (back-to-back position⁴⁷). The essential tetramer model of AMPA GluR2 LBD was built by protein–protein docking with SymmDock³³ (see the Supporting Information for the table with results). The relative position of dimers in a tetramer was modeled by consideration of the experimentally measured dimensions of the receptor^{48–51} as well as the 2-fold symmetry. Among the 100 generated docking results only 1 GluR2 LBD tetramer (identified among the first 20 models) fulfilled the above requirements, i.e. result 14 was ranked according to the geometric shape complementarity score, 11812 (see the Supporting Information for the table with results). This result was characterized with a high value of the approximate interface area of the complex (1667.00) and atomic contact energy (529.94). Next, Modeller9v1³² was applied for the generation of a population of 100 models of the GluR6 LBD tetramer, and the highest-scored model was

selected. Water molecules were added to the model according to the AMPA GluR2 receptor (PDB code 1FTL⁴⁶) template. The final model of the GluR6 LBD tetramer has dimensions of 116 Å × 72 Å, which is in accordance with single-particle electron microscopy images.^{48–51}

In the next step of studies the model of the GluR6 receptor transmembrane region was constructed. KcsA potassium channels (PDB codes 1K4C⁵² and 1JVM⁵³ for a subunit and a channel, respectively) were applied as templates, similarly to the methodology of Zefirov's research group^{12,13} and our previous work on the GluR5 receptor.¹⁵ The sequence identity between the target and the KcsA potassium channel template was 18.56%. The M3 helices in the opposite subunits were manually bent with Spdbv⁴⁵ (with the Ramachandran plot control) with the same angle on the first serine residues from the SSYTANLAAF motif (and refined by Modeller9v1³²), resulting in the orientation of the M3 helices in the neighboring subunits in the opposite directions as indicated by the experimental results of Sobolevsky et al.⁵⁴ and described earlier for the GluR5 receptor.¹⁵ Similarly, the M4 helix was constructed and added to the model according to the previously described methodology.¹⁵

The next step of research was the building of models of linkers connecting the transmembrane region and the ligand binding domain. Analogously as for the GluR5 receptor, the S1–M1 linker of the GluR6 protein was modeled with the application of ab initio Rosetta³⁴ methodology and refined with the Modeller9v1³² dope-loopmodel module, and the M3–S1 linker was built with Modeller9v1,³² whereas the S2–M4 linker was constructed with Biopolymer ModelLoop incorporated in Sybyl7.3 and refined with the Modeller9v1³² dope-loopmodel module. This resulted in a model of the GluR6 receptor consisting of a transmembrane region connected via the necessary linkers with the ligand binding domain. The proper symmetry of a model was ensured by a suitable module of Modeller9v1.³²

The third, N-terminal domain was modeled in a similar manner as the ligand binding domain. The metabotropic mGluR3 receptor (PDB code 2E4U⁵⁵) was selected as a template. The sequence identity between the target and the template was 22.72%. mGluR3 receptor coordinates were used as an input for the SymmDock³³ server to construct a suitable tetramer (see the Supporting Information for the table with results). Among 100 generated docking results, result 7 (ranked according to geometric shape complementarity score) was selected with the score of 20264, and one of the highest values of atomic contact energy equaled 1271.70. This result was characterized by the proper 2-fold symmetry and ensured the shape and the dimensions of a GluR6 receptor model which are in accordance with the experimental results. The model of the mGluR3 receptor tetramer

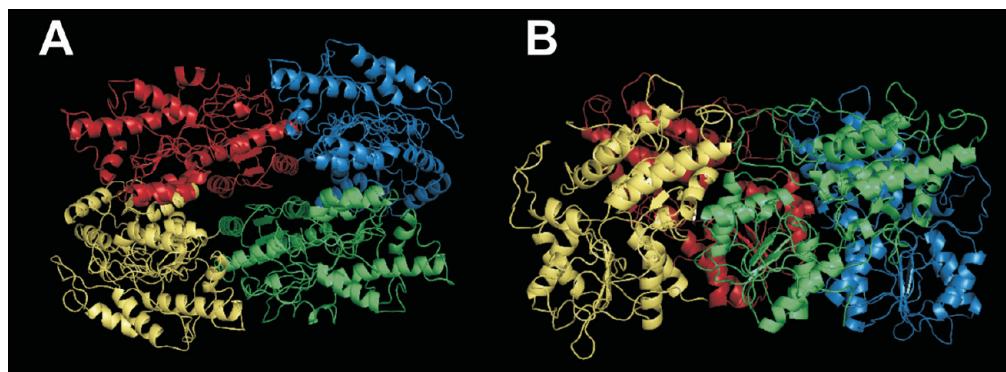


Figure 3. The model of the GluR6 N-terminal domain tetramer. A - top view; B - side view. Each subunit is represented by a different color.

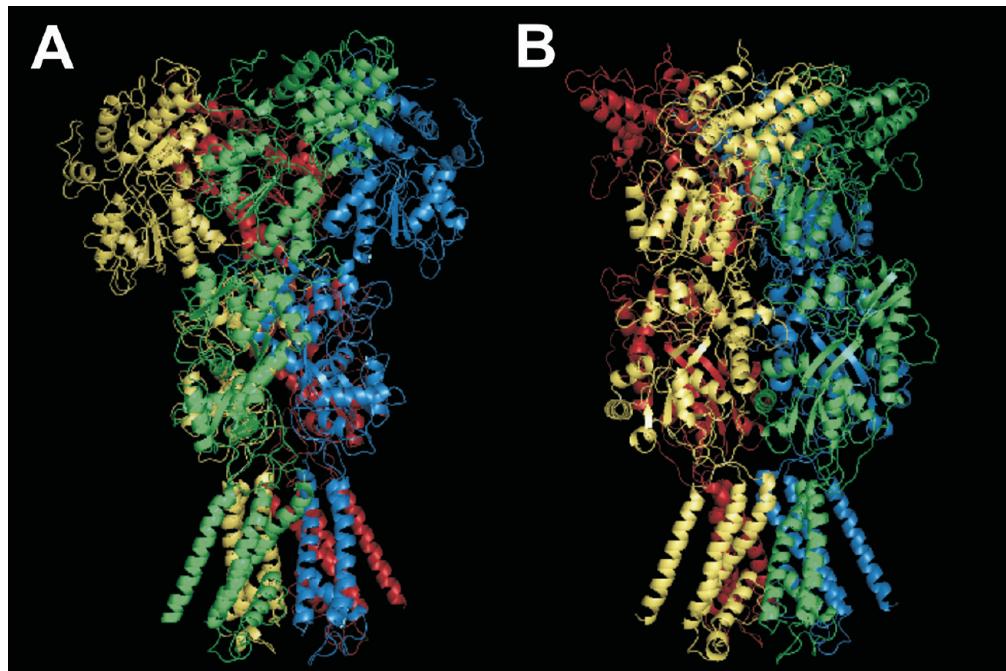


Figure 4. The final model of the GluR6 receptor, A, B - side view. Each subunit is represented by a different color.

was applied to construct a model of the GluR6 receptor N-terminal domain with Modeller9v1.³² Again a population of 100 models was generated, and the highly scored model was selected as the final one (Figure 3, A-B). The linkers connecting the N-terminal domain and the ligand binding domain were constructed with the application of Biopolymer ModelLoop incorporated in Sybyl7.3 and refined with the Modeller9v1³² dope-loopmodel module. These linker models were used to combine NTD and LBD, which resulted in a complete model of the GluR6 receptor. The proper symmetry of the final model was ensured by a suitable module of Modeller9v1.³²

Finally, the GluR6 receptor model was refined with application of Yasara Dynamics.³⁵ First, the conformations of side chains were minimized with the Yamber3 force field at default parameters. Then, the GluR6 receptor model was placed in a water box (in the region of transduction domain only) to enable subsequent procedures of molecular dynamics run.¹⁵ The MD run of water molecules with the fixed receptor protein was followed by allowing free conformational changes of side chains of the residues constituting transduction domain and finally - by MD simulation of the free linkers. The procedure of receptor refinement with molecular

dynamics was applied to the transduction domain only as - first - this part of the GluR6 receptor model was constructed with ab initio methods and second - novel noncompetitive GluR6 antagonists were docked to this domain.

The final GluR6 receptor model (Figure 4) has dimensions of 159 Å × 118 Å × 117 Å which, similarly as the receptor shape, is in perfect agreement with the experimental results.^{48–51}

Each of the four subunits of the GluR6 receptor model is stabilized by a network of salt bridges and hydrogen bonds. Formation of the tetramer in the NTD domain is enabled by two salt bridges: Lys190A-Glu223C and Lys216C-Asp143B. The LBD tetramer is also stabilized by a few intersubunit salt bridges, which for subunit A involve the following: Glu524A-Lys531B, Lys536A-Glu524B, Glu686A-Lys451D, Asp703A-Arg748D, and Arg775A-Asp776B. Moreover, there are two intersubunit salt bridges connecting LBD and the transduction domain: Lys704A-Glu806D and Lys704C-Glu806B. The transmembrane region is stabilized by the intersubunit interaction of side chains, namely, a hydrogen bond between Asn610 from the P loop and Arg633 from the M3 helix.

Table 1. Docking Results for the GluR6 Receptor Competitive Antagonist

Compound	Formula	Total	Crash	Polar	D	PMF	G	Chem	C	K _i ³⁷
		Score			Score	Score	Score	Score	Score	μM
LU 97175		10.38	-2.05	5.28	-136.28	-100.94	-255.28	-32.53	5	3.11

Table 2. Docking Results for the GluR6 Receptor Uncompetitive Blocker

Compound	Formula	Total	Crash	Polar	D	PMF	G	Chem	C	K _i
		Score			Score	Score	Score	Score	Score	
IEM-1754		8.48	-1.83	5.39	-149.40	-0.85	-243.90	-31.93	4	Not available

2. Validation of the Constructed GluR6 Model by Docking of Competitive and Uncompetitive Antagonists.

The methodology used for the GluR6 receptor model construction was validated by the docking of a competitive antagonist, LU 97175,⁵⁶ **4** (compare Table 1), and an uncompetitive antagonist, IEM-1754,⁵⁷ **5** (compare Table 2). The methodology used for construction of the NTD tetramer was not validated as there are not known kainate receptor ligands which interact with this domain. Such ligands have been identified for NMDA receptors only. Furthermore, as it was stressed in the Introduction section, NTD dimer models have been already constructed,^{8–10} and corresponding docking studies have been performed⁹ for ligands interacting with the dimer interface. Thus, this part of the methodology may be treated as previously validated. As there are not known ligands for any iGluRs which bind on the NTD tetramer interface (similarly as in the case of the LBD tetramer interface), no validation was possible for this new part of the procedure.

The docking of **4** to the GluR6 receptor was performed with Surflex⁴¹ incorporated in Sybyl7.3. This procedure was guided by the docking of this ligand to the AMPA receptor LBD dimer (crystal structure with DNQX, PDB code 1FTL⁴⁶). The interactions of **4** with the GluR6 receptor correspond to the way of interaction of DNQX with the AMPA GluR2 receptor in the crystal structure (compare Figure 5, A-B). Two keto groups in the quinoxaline system interact via hydrogen bonds with Arg523 of the S1 lobe. One of these groups additionally forms a hydrogen bond with a water molecule, whereas the other one - with the hydrogen atom of the peptide bond of Ala518. The quinoxaline NH

hydrogen atom forms a hydrogen bond with the carbonyl oxygen atom of Pro516. The network of hydrogen bonds is completed by the interaction of the amide moiety of the ligand with another water molecule. Several other water molecules are present in the binding pocket and form an internal network of hydrogen bonds stabilizing the protein and simultaneously completing the binding pocket. Tyr520 is engaged in $\pi-\pi$ interaction with the quinoxaline system of **4**. The hydrophobic pocket is formed by Pro516 and Ala518 from S1 and Val685 and Met737 from S2. Table 1 contains scoring function values for the highest-scored ligand pose.

Uncompetitive antagonists of iGluRs bind in the transmembrane region of a receptor. Interactions of **5** with the GluR6 receptor follow the manner of interactions of this ligand with the GluR5 receptor.¹⁵ In the obtained receptor-ligand complex (see Figure 6, A-C) the protonable nitrogen atoms of the ligand form hydrogen bonds with all four Gln621 in the four subunits. The protonable nitrogen atom at the end of the alkyl chain interacts with peptide bond oxygen atoms, whereas the other protonable nitrogen atom (closer to adamantane moiety) interacts with side chains of Gln621 from two neighboring subunits. The alkyl chain of **5** is placed in the crevice constituted by the side chains of Gln621 from the four subunits of a channel. The main hydrophobic group of **5**, i.e. the adamantane moiety, is surrounded by the side chains of Leu645 and Ile648. Moreover, Ser649 and Thr652 properly close the binding pocket at the pocket top part. Table 2 contains scoring function values for the highest-scored ligand pose.

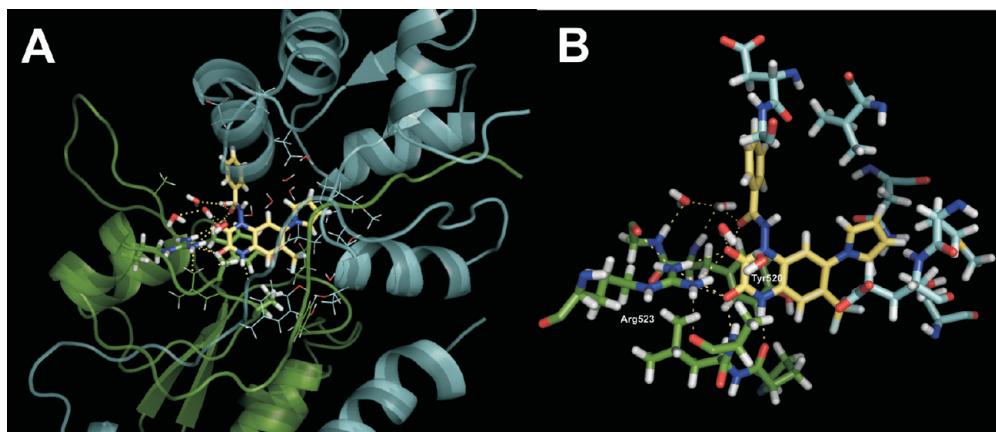


Figure 5. LU 97175 in the binding pocket in LBD of the GluR6 receptor. A - an overview of the binding pocket on the LBD dimer interface; B - the detailed view of the binding pocket.

3. Identification of a Binding Site for Novel GluR6 Noncompetitive Antagonists and Ligand Docking. There are no literature reports available concerning the manner of interaction of kainate receptor noncompetitive antagonists with their molecular targets. Thus, the studies described below were guided by experimental results by Balannik et al.²¹ who proposed the interaction model for AMPA receptor noncompetitive inhibitors.

The starting point for their consideration was the hypothesis that allosteric interaction takes place between the binding sites of cyclothiazide (AMPA receptor positive modulator) and investigated AMPA receptor noncompetitive antagonists (GYKI 53655 and CP465,022). In the first series of experiments they stated that both GYKI 53655 and CP465,022 bind with the higher affinity to the receptor in the closed (resting) or desensitized state. When ligands of such a type bind to the receptor in the open state, the affinity of a negative modulator is reversely proportional to the potency of an agonist. It probably results from the fact that binding of an agonist in the LBD and subsequent closure of this domain cause significant conformational changes in the binding site of noncompetitive antagonists. This observation formed the base for the second part of their studies which involved verification of a hypothesis that the binding site for AMPA receptor noncompetitive antagonists is situated in the transduction domain. After a series of experiments they concluded that the binding pocket of AMPA receptor noncompetitive antagonists is situated between S1-M1 and S2-M4 linkers belonging to the neighboring receptor subunits.

Searching for the binding site for novel indole-derived noncompetitive GluR6 receptor antagonists¹⁷ was facilitated by the fact that they have significantly lower affinity toward GluR5 receptor. Thus, it was decided to look for sequence differences between GluR5 and GluR6 receptors S1-M1 and S2-M4 linkers. Figure 7 depicts sequence alignment of these fragments of GluR5 and GluR6 receptors. It can be noticed that there is no difference between the sequences of GluR5 and GluR6 S1-M1 linkers. However in the S2-M4 linker of the GluR6 receptor the sequence Glu808-Ser809 corresponds with Asp823-Asn824 in the GluR5 protein. It was thus concluded that Ser809 in GluR6 and Asn824 in GluR5 must be crucial residues for ligand binding.

Two novel ligands, the lead structure **6** and about 10 times less active derivative **7** (compare Table 3), were docked to the GluR6 receptor model with Surflex.⁴¹ The presence of

water molecules was not considered for the transduction domain because there are not any suitable templates (or other experimental data) to add them correctly. The following residues were recognized as key for interactions: Arg663A, Arg663B (M3-S2 linker), Ser809B (S2-M4 linker), and Phe553A (S1-M1 linker). The binding cavity is closed properly by Ile755A, Pro558B, and Leu559B. In the case of lead compound **6** (Figure 8, A-B and Figure 9, A-B) Arg663A, Arg663B, and Ser809B are engaged in the formation of hydrogen bonds with methoxyl groups of the ligand. Phe553A may interact via π - π interaction with the aromatic ligand moiety. In the obtained ligand–receptor complex this interaction is only potential, but this is justified by the methodology used for receptor model building. It is generally agreed that the protein model is suitable for docking purposes when the sequence identity between the protein and the applied template is not lower than 60%. However, because of the high lipophilicity of novel indole derivatives (with logP values in the range of 4–5), it may be concluded that hydrophobic interactions are important for formation of ligand–receptor complexes in this case. The derivative **7** possesses only one methoxyl group which forms a hydrogen bond with Arg663A. In the case of both ligands the binding pocket is closed properly by Ile755A, Pro558B, and Leu559B.

The performed docking procedure of novel noncompetitive GluR6 receptor antagonists was simultaneously - to a certain extent - a validation of methodology used for GluR6 receptor model building as it confirmed that transduction domain, constructed partially by ab initio methods, was built correctly.

DISCUSSION

Noncompetitive antagonists of ionotropic glutamate receptors are promising medicinal substances. Especially ligands of AMPA and kainate receptors are worth investigating as they do not tend to exhibit psychotomimetic effects, traditionally linked with high affinity NMDA receptor blockers. However, as it was stressed earlier, there are few known kainate receptor ligands of that type. Construction of the GluR6 receptor model and identification of a binding site for noncompetitive inhibitors described in this paper will facilitate discovery of novel active compounds.

The presented GluR6 receptor model is the first complete model of any ionotropic glutamate receptor. It is in agreement with all the available experimental data, including the

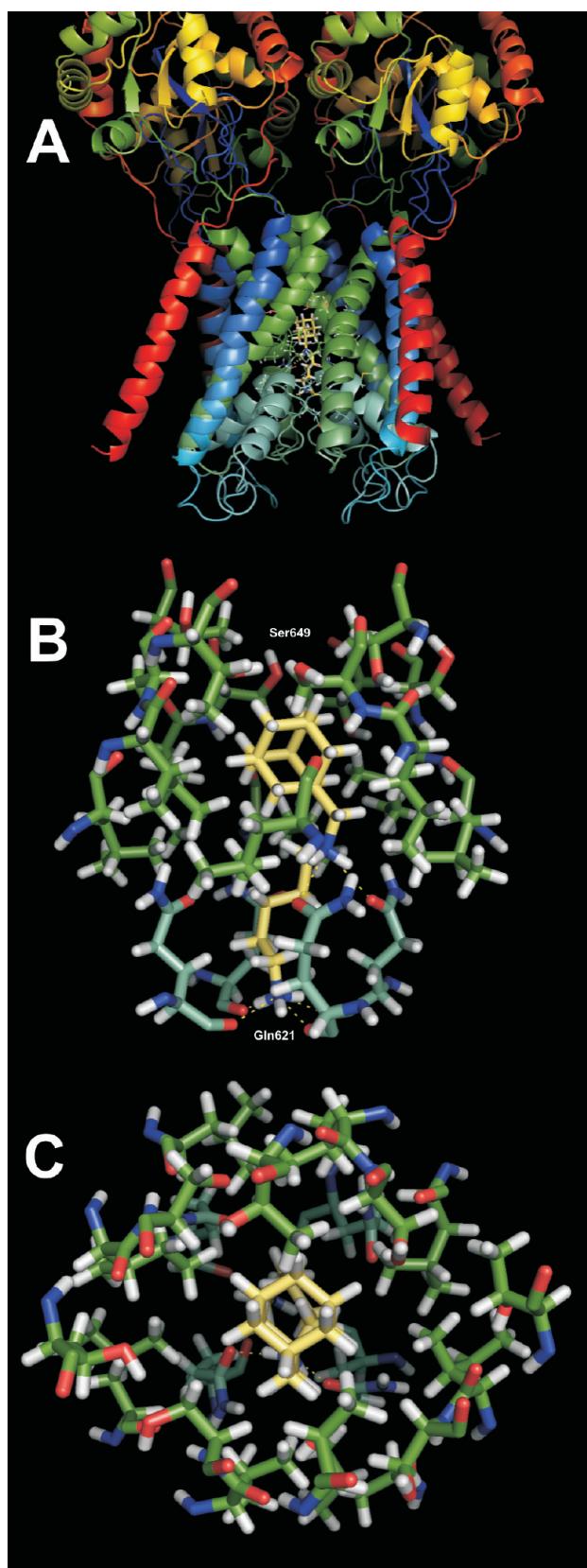


Figure 6. IEM-1754 in the binding pocket of the GluR6 receptor. A - an overview of the binding pocket; B - the detailed side view of the binding pocket; C - the detailed top view of the binding pocket.

electron microscopic model, obtained by Madden's group,⁴⁸ depicted in Figure 10. Madden's group model constitutes the first direct evidence for the overall 2-fold symmetry of the iGluRs, supporting a dimer-of-dimers molecular archi-

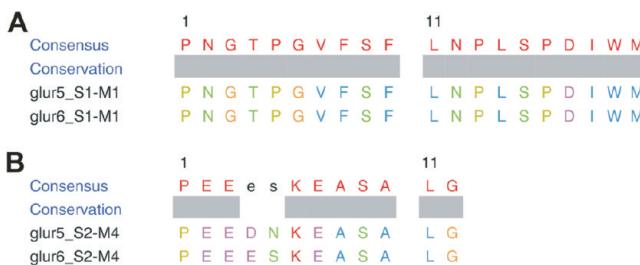


Figure 7. The sequence alignment of GluR5 and GluR6 receptor in the region of transduction domain (S1-M1 linker and M3-S1 linker) performed with MAFFT.³¹

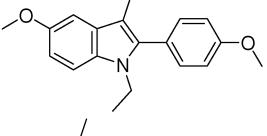
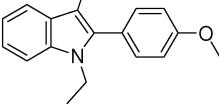
ture,⁴⁸ and it is in agreement with earlier studies on the association properties of iGluR domains and on the electrophysiological properties of iGluR subunits. According to their model, iGluRs are approximately oblate rotational ellipsoids with 2-fold symmetry along their longitudinal axes and overall dimensions of 170 Å × 140 Å × 110 Å. Furthermore, they distinguished four “arms” of the receptor tetramer which run between the bottom and top parts (Figure 10). These “arms” are located around the central vestibule with a helical twist, and two of the “arms” are thicker and longer than the other two.⁴⁸ Figure 10 depicts that each short “arm” merges with one long “arm” before linking with the top; a second lateral connection links each short “arm” to the other long “arm”.⁴⁸ Comparison of Madden's group model and the presented model of the GluR6 receptor reveals that the general shape of both models is very similar. As it was stressed earlier the dimensions of the GluR6 model (159 Å × 118 Å × 117 Å) are in agreement with the dimensions of Madden's group model, taking into account its resolution of 20 Å.

Each subunit of the constructed GluR6 model contains a cysteine bridge between the ligand binding domain (Cys750 from S2 lobe) and the transduction domain (Cys804). The function of these cysteine bridges may be analogous to the importance of nine conserved cysteine residues forming three predicted disulfide bridges in metabotropic glutamate receptors which could function as a spring between the ligand-binding domain and the intracellular signaling mechanism connected to the TM regions.^{57,58}

The proposed model of the GluR6 receptor transduction domain is consistent with the model by Speranskiy and Kurnikova¹⁶ in such a way that both models reproduce S1-M1 and M3-S2 linkers by coil structures, stabilized by salt bridges and hydrogen bonds. Moreover, in both models the S1-M1 linker is a loop structure. However, Speranskiy and Kurnikova¹⁶ listed different stabilizing interactions, probably because they constructed a model of only a small part of a receptor and did not test it by docking studies. In the proposed model the S1-M1 linker is stabilized by three hydrogen bonds in which main or side chains of Pro545 and Thr548, Ser554 and Asn557, and Pro561 and Ile563 are involved. The M3-S2 linker is stabilized by a salt bridge of Arg663 as indicated also by Speranskiy and Kurnikova.¹⁶ In the presented model, however, it forms an intersubunit bond with Glu665 from a neighboring subunit.

The linkers between NTD and LBD may constitute another transduction domain in the receptor and participate in the transmission of the signal between NTD and LBD. The residues involved in the signal transmission are most probably the following: Lys777D-Tyr195B-Asp426D. As the

Table 3. Docking Results for GluR6 Receptor Noncompetitive Antagonists

Compound	Formula	Total	Crash	Polar	D	PMF	G	Chem	C	IC_{50}^{17}
		Score	Score	Score	Score	Score	Score	Score	Score	μM
6		5.68	-1.75	1.73	-129.00	-53.91	-197.86	-27.19	5	0.7
7		4.65	-1.46	1.03	-160.84	-51.31	-186.29	-21.49	5	10.0

N-terminal domain participates in allosteric modulation of NMDA receptors, it could be expected that it may also constitute binding sites for noncompetitive antagonists of AMPA and kainate receptors. The constructed model of GluR6 receptor will facilitate such studies in the future.

The methodology applied in the GluR6 model construction was validated by docking of a competitive GluR6 antagonist, LU 97175, and an uncompetitive blocker, IEM-1754.

The docking pose obtained for a competitive antagonist **4** is in agreement both with the pose of DNQX in the AMPA

receptor LBD crystal structure and extensive literature data. Up to now multiple crystals of binding domains of ionotropic glutamate receptors have been resolved. In all the solved structures an arginine side chain from D helix creates the major anchor point for the α -carboxylic group of the ligand. This is a key residue for high affinity binding of glutamate or glycine (in the case of the NMDA receptor NR1 subunit). Even when this residue is mutated to lysine, the agonist affinity is significantly decreased. Binding of the α -amino group of the ligand occurs through a conserved glutamate

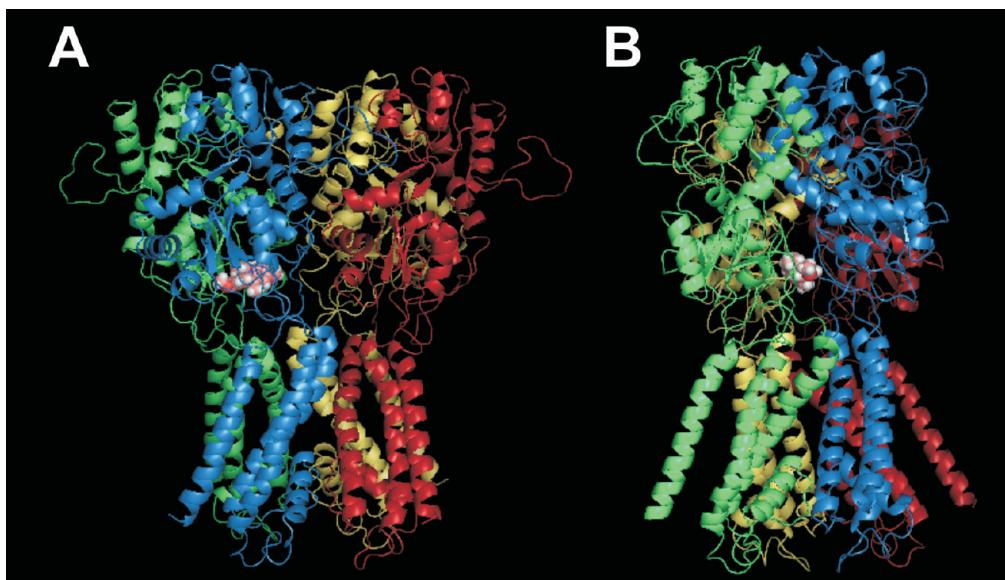


Figure 8. Novel noncompetitive antagonist, **6**, in the GluR6 receptor. A, B - side view. N-terminal domain not shown.

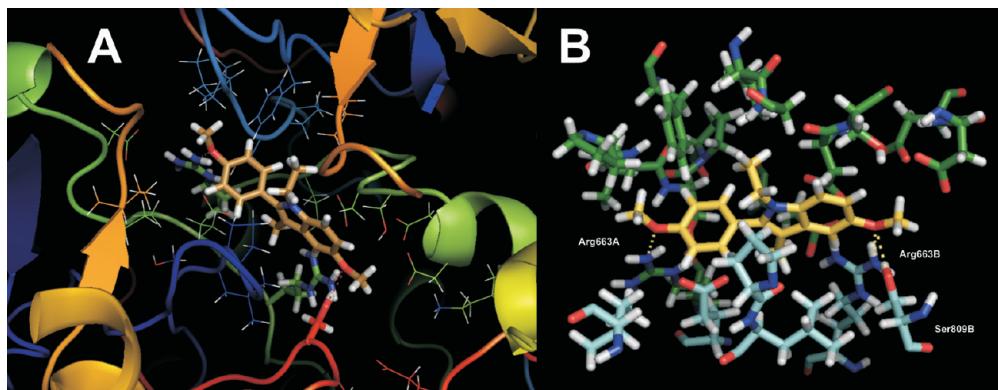


Figure 9. Novel noncompetitive antagonist, **6**, in the binding pocket of the GluR6 receptor. A - an overview of the binding pocket on the transduction domain dimer interface; B - the detailed view of the binding pocket.

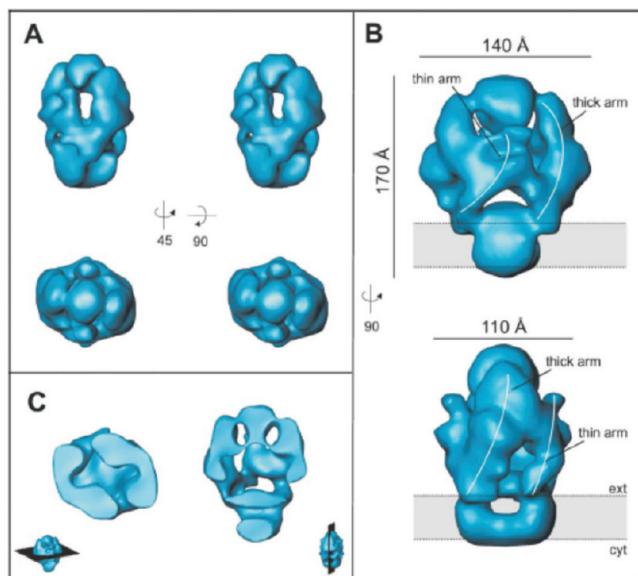


Figure 10. Representations of the 3D structure of the GluR6 ionotropic glutamate receptor: A - stereo views; B - orthogonal side and front views; C - cut-away views. The lower stereopair in (A) represents a top view of the molecule as it would appear looking down on the membrane from the synaptic cleft. The upper stereopair in (A) shows a side view of the molecule, which is offset 45° around a vertical axis relative to the views shown in (B). In (B), the proposed location of the membrane is indicated by a shaded rectangle at the bottom of each figure, perpendicular to the receptor's longitudinal, 2-fold symmetry axis and perpendicular to the image plane. Ext. - extracellular; cyt. - cytosolic; the thickness of the membrane as drawn is 36 Å. In (C), horizontal and vertical cut-away views of the central vestibule are shown, together with inset representations of the corresponding cutting planes. Figure reprinted with permission of Elsevier from ref 48, copyright Elsevier 2004.

side chain in the case of AMPA and kainate receptors and by an aspartate for NMDA receptors. The γ -carboxylic group of the ligand makes hydrogen bonds with the main-chain peptide bond and the hydroxyl group of a conserved threonine side chain. The key residue, Arg523, in GluR6 corresponds with Arg615 in GluR2. For the full interactions with ligands there are also four water molecules necessary which create a network of stabilizing hydrogen interactions which is also confirmed by our docking results.

The docking results for IEM-1754 are consistent with a commonly acceptable model of iGluR channel blockers interaction which indicates the N/Q/R site as the main anchoring point. The obtained docking results are also consistent with site-directed mutagenesis data.⁵⁹ These follow the idea of the previous studies of Magazannik's group¹³ as the ligands were placed in the center of a channel vestibule.

The detailed discussion of the uncompetitive blocker docking to iGluRs as well as the model of channel gating are available in ref 15.

The functioning of ionotropic glutamate receptors, including kainate receptors, may be described by the MWC allosteric model (from the names: Monod, Wyman, Changeux). According to this model there is a dynamic equilibrium between three conformational states of a receptor, i.e. resting, open, and desensitized states (Figure 11). This equilibrium is affected by multiple external factors, including temperature and membrane potential. It also depends on the presence of allosteric modulators. Allosteric modulators stabilize a receptor in one of its conformational states. The MWC allosteric model allows for explaining the potential existence of multiple sites of allosteric modulators on iGluRs. Indeed, several allosteric binding pockets of NMDA and AMPA receptors have already been identified. Allosteric modulation of NMDA receptors occurs via transmembrane or NTD domain. In the case of AMPA receptors, this occurs through transmembrane domain, transduction domain, or - in the case of positive modulators, like cyclothiazide or aniracetam through the site in the LBD different from the agonist binding pocket. However, there are noncompetitive antagonists of AMPA receptors, like a dye, Evan's blue, which do not bind in any of the known sites of allosteric modulation.

It is worth emphasizing that all the identified allosteric binding sites, with the exception of the pocket for uncompetitive antagonists, are situated on the dimer interface of one of the domains (NTD, LBD, transduction domain). This stays also true for the proposed binding pocket for kainate receptors noncompetitive antagonists.

The docking results of indole-derived noncompetitive antagonists to the GluR6 receptor allows for confirming and widening suggestions of Balannik et al.²¹ concerning the mechanism of the action of iGluR noncompetitive blockers of that type. Such ligands stabilize a glutamate receptor in the closed state, and - through the blockade of movement in the region of transduction domain - they make impossible transduction of the signal from the LBD to the transmembrane region. According to our model, the M3-S1 linker is crucial for this process as the M3 helix is the most important one for the process of gating. Recently, Ritz et al.⁶⁰ investigated the mechanism of inhibition of AMPA channels by 2,3-benzodiazepine derivatives. They stated that binding of an inhibitor to the receptor involves formation of a loose, partially conducted channel intermediate, which rapidly isomerizes to a tighter complex.⁶⁰ Our model reflects the second step of this process.

It may be expected that water molecules are important for binding of noncompetitive inhibitors in the iGluR transduc-

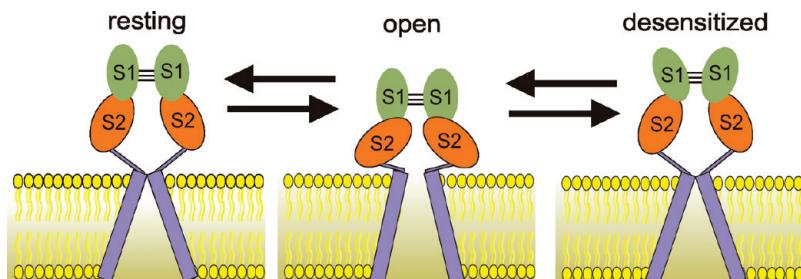


Figure 11. Conformational states of ionotropic glutamate receptors (NTD not shown).

tion domain, similarly as it is for the ligands binding in the region of LBD (agonists, competitive antagonists, and positive modulators). However, it was impossible to build a reliable model of the transduction domain including water molecules because of the lack of suitable templates.

The constructed model of the GluR6 receptor allowed for describing the 2-fold receptor symmetry on the molecular level. It is particularly visible in the case of interactions of uncompetitive channel blockers with the transmembrane region. The model allowed for identifying a novel binding pocket for GluR6 receptor noncompetitive antagonists and explaining the mechanism of action of these compounds on the molecular level. The characteristics of the novel binding site will enable the designing of more active noncompetitive antagonists for all three types of iGluRs. Moreover, the construction of the NTD model may bear fruit of any AMPA/kainate receptor inhibitors interacting with this domain.

CONCLUSIONS

The presented model of the GluR6 receptor is the first complete model of any iGluRs. It exhibits the correct symmetry: 2-fold in the N-terminal domain, ligand binding domain, and an external part of the transmembrane region, whereas it is 4-fold deeper in the channel. The shape and dimensions of the model are in accordance with the available experimental data including single-particle electron microscopy results. The constructed model allowed for indicating the residues engaged in salt bridges stabilizing the tetramers of particular domains. Moreover, it was proposed that the cysteine bridge between the ligand binding domain (Cys750 from S2 lobe) and transduction domain (Cys804) may be important for signal transduction from LBD to the transmembrane region. Although corresponding cysteine bridges are present only in a fraction of solved crystal structures of LBD, it may result from the applied method of crystallization. The methodology of the GluR6 receptor model building was validated in the procedure of docking of competitive and uncompetitive antagonists. The constructed model enabled the identification of a novel allosteric binding site on the GluR6 receptor. Following experimental studies by Balannik et al., it was suggested that noncompetitive antagonists of kainate receptors bind on the dimer interface in the transduction domain. The mechanism of receptor blockade by compounds of such a type involves stabilization of the receptor in the closed state by the prevention of linkers (especially M3-S1 linker) movement in the transduction domain.

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CTD, C-terminal domain; iGluR, ionotropic glutamate receptor; KA, kainic acid; LAOBP, lysine-arginine-ornithine binding protein; LIVBP, leucine-isoleucine-valine-binding protein; LBD, ligand-binding domain; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; NTD, N-terminal domain.

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Supporting Information Available: Tables with (1) SymmDock results for LBD and NTD tetramers; (2) values of angles of GluR6 receptor residues constituting the binding cavity for LU 97175; (3) values of angles of GluR6 receptor residues constituting the binding cavity for IEM-1754; and (4) values of angles of GluR6 receptor residues constituting the binding cavity for novel noncompetitive GluR6 receptor antagonists, **6** and **7**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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