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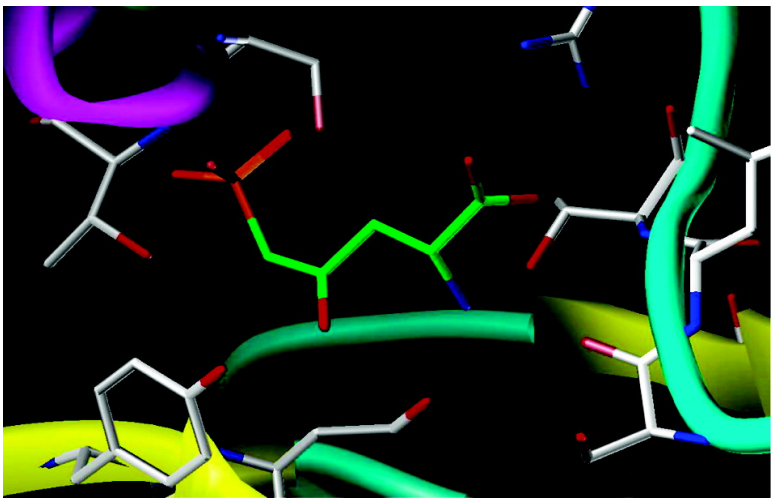

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Development of a Three-Dimensional Model for the *N*-Methyl-D-aspartate NR2A Subunit

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NR2 subunits of *N*-methyl-D-aspartic acid (NMDA) receptors are known to bind the neurotransmitter glutamate, competitive agonists, and antagonists. Since crystallographic data of these proteins are not available, we built a homology model of the ligand binding domain of the NR2A subunit. A consensus binding mode of selected AP5-like NMDA antagonists has been ascertained using molecular docking. The present 3D model gives insights for the design of new NMDA subtype selective compounds.

Introduction

N-Methyl-D-aspartic acid (NMDA) receptors belong to the ionotropic glutamate receptor family with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. These ion channels are gated upon the binding of the endogenous glutamic acid [(*S*)-Glu, Figure 1] and are involved in several physiological functions such as memory, learning, and synaptic plasticity. Hyperactivation of the NMDA receptors can cause neurodegenerative pathologies such as Parkinson's and Alzheimer's diseases and amyotrophic lateral sclerosis. The most common strategy to counteract the hyperactivation of glutamatergic pathways is the use of the NMDA receptors antagonists.[1] Nevertheless, clinical trials with NMDA antagonists brought about the occurrence of several adverse central nervous system (CNS) effects, including hallucinations, a centrally mediated increase in blood pressure, and at high doses catatonia and anesthesia.[2−4]

NMDA receptors are heteromeric assemblies of different subunits. In fact, recent experimental data show that three different subunits, NR1, NR2, and occasionally NR3, form the native heterotetrameric NMDA receptors.[7−12] Early electrophysiological studies[9] demonstrated that the NMDA receptor activation requires occupation of the glycine binding site localized into the NR1 subunits, while opening of the gates is triggered by the binding of glutamate into the NR2 subunits. Conversely, the NR3 subunits, occasionally present in two variants (A and B) in the NMDA receptor assemblies, seem to inhibit the channel activity.[12] Moreover, the NR1 subunits are expressed in eight different splicing variants, whereas the NR2 subunits exist as four isoforms (NR2A−D) encoded by four different genes. This array of variants is responsible for the functional and regional heterogeneity of NMDA receptors, thus influencing the pharmacological properties of this receptor complex in the CNS.[13] The NR2A/C subunits were mainly found in the cerebellar granule cell layer, whereas the NR2D subunit is predominant in the diencephalons. The NR2B subunit, occasionally associated with the NR3 subunit, mediates the glutamate effect in the medial striatum. The proteins of the ionotropic glutamate receptor family share the same topology. Visual inspections of available crystal structures of this family identified the ligand binding site in the cleft between the two soluble regions termed S1 and S2.[14,15] S1 constitutes part of the region preceding the transmembrane (TM) α-helix 1 (TM1), whereas S2 is the extracellular domain between TM3 and TM4. Moreover, site-directed mutagenesis studies of Anson[16] and Lummis[17] identified the amino acids that play a crucial role in the activation of the NR2A subunit. Their replacement brings about a 4- to 1000-fold reduction in binding affinity for both glutamate and (*R*)-AP-5. Owing to the heterogeneity at the molecular level and the different CNS localization of the NMDA subunits, the discovery of new subunit selective ligands[5,6] is the goal of an updated research project in the field.[11] A reliable molecular model for each subunit could be a valuable tool to rationally discuss the structure−activity relationships of the large number of NMDA antagonists and to design new subtype selective ligands.

In this paper, the homology modeling of the rat NR2A subunit soluble domains (S1−S2) and the results obtained by molecular docking of some (*R*)-AP5-like NMDA antagonists into the new model are reported.

Results and Discussion

On the basis of the GluR2/S1−S2 X-ray crystal data, Armstrong and Gouaux[14] made clear that AMPA and glutamate, which are full agonists sequestered in the receptor's cleft, induce about 20° of S1−S2 domain closure by promoting a rearrangement of the protein backbone between Asp651 and Gly653. Conversely, kainate, a partial agonist at the AMPA receptors, induces 12° of domain closure only. This evidence supports the hypothesis[14] that the ligand binding domain of AMPA receptors may exist in a range of states

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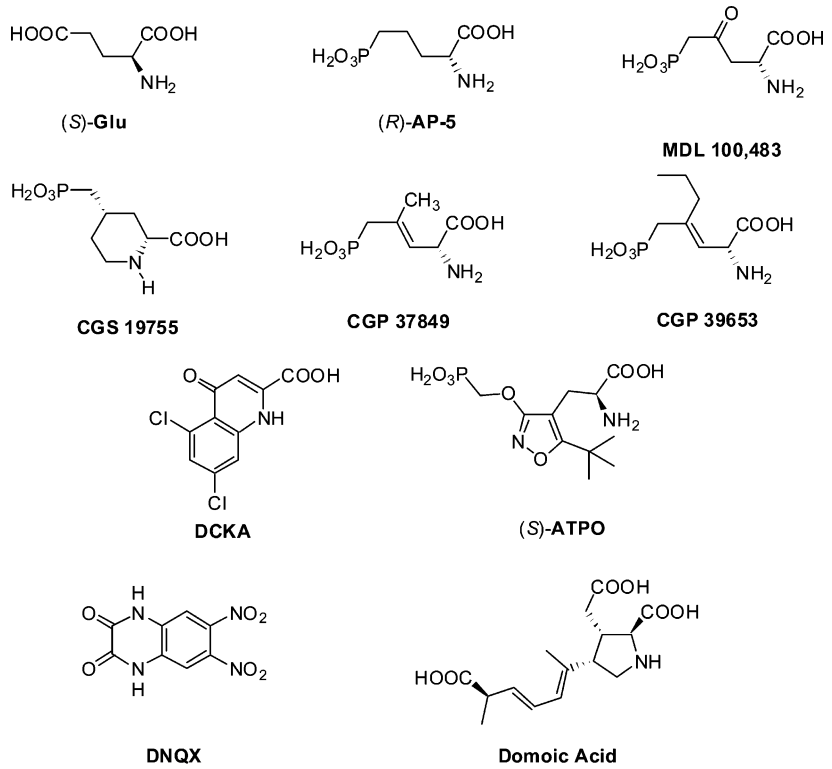

Figure 1. Structures of glutamic acid and related ligands.

(different degrees of domain closure) between the agonist-bound state (closed form) and the antagonist-bound state (open form), which is similar to the Apo state. As a consequence, ligands bulkier than glutamate act as antagonists, since they stabilize the open form, while ligands similar in size to glutamate act as agonists by stabilizing the closed form. Consistent with the AMPA/GluR2 interaction, the NMDA/NR1 complex displays the same activation mechanism.[15]

The comparable structural requirements for the NMDA and AMPA ligands and the high sequence homology between the NR1 and the NR2 subunits suggest that the ligands of the NMDA receptors might operate as the AMPA ligands do. To validate this hypothesis, we built a homology model of the NR2A/S1−S2 domain using as templates both the ligand binding domain of NMDA and AMPA subunits cocrystallized with selective antagonists: (a) GluR2/S1−S2 cocrystallized with DNQX/sulfate (DNQX = 6,7-dinitroquinoxaline-2,3-dione, PDB[18] code 1ftl)[14]); (b) GluR2/S1−S2 cocrystallized with (*S*)-ATPO ((*S*)-2-amino-3-[5-*tert*-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid, PDB code 1n0t[19]), and (c) the NR1/S1−S2 construct with DCKA (5,7-dichloro-4-hydroxyquinoline-2-carboxylic acid, PDB code 1pbq[15]).

As the initial step of the comparative modeling, the amino acid sequence corresponding to the target soluble domains (S1 and S2) was isolated. With few cycles of iterative alignment between the protein encoded by the complete NR2A-related gene, GRIN2A, and the sequence of the known templates, the highest homology was found for two different portions: S1 corresponds to the segment H405-R539, while S2 is the K669-C800 portion (Figure 2). Incorporation of the GT linker completed the sequence of the model and allowed us to gain an optimized homology with the GluR2/S1−S2 and NR1/S1−S2 template.

Alignment of the NR1/S1−S2 and NR2A/S1−S2 sequences showed 36% identity (Figure 2). The comparison of the GluR2/S1−S2 with NR2A/S1−S2 sequences indicated a 26% identity and the absence of a 24 amino acid segment in the AMPA subunit corresponding to the amino acid string N432-C455 in the NR2A sequence (Figure 2).

Considering that an identity of 26% is able to produce a valuable model[21] and that the NR1 endogenously binds glycine, which is a smaller molecule than glutamate and therefore the binding site may be too small and closed for accommodating any of the antagonists that are known to bind more likely to a more "open" conformation,[14,15] the NR2A model derived from the GluR2 templates was built. The superimposition of the two templates GluR2/S1−S2 (DNQX) and GluR2/S1−S2 (ATPO) revealed that they are nearly identical with an rmsd of 0.6 Å for their protein backbone atoms. This value is clearly within the accuracy of the reported crystal structures with resolution of 1.8 and 2.1 Å, respectively. The GluR2/S1−S2 (ATPO) was finally chosen as the template because the ligand induces, in the open form, minor conformational changes than DNQX, and consequently, this template is suited to better reproduce the antagonist-bound conformation.[19] Moreover, (*S*)-ATPO shares with the NMDA selective antagonists closer structural features (Figure 1), and this evidence could support the hypothesis of the analogy of their reciprocal binding modes. The 3D comparative model of our target was obtained after elaborating the NR2A sequence and the PDB coordinates of the selected template with the SWISS-MODEL server, described in the Methods and Experimental Section.

The stereochemical accuracy of the model was checked by means of PROCHECK3.0[22,23] and VERIFY3D[24,25] software. To evaluate the consistency of the model with

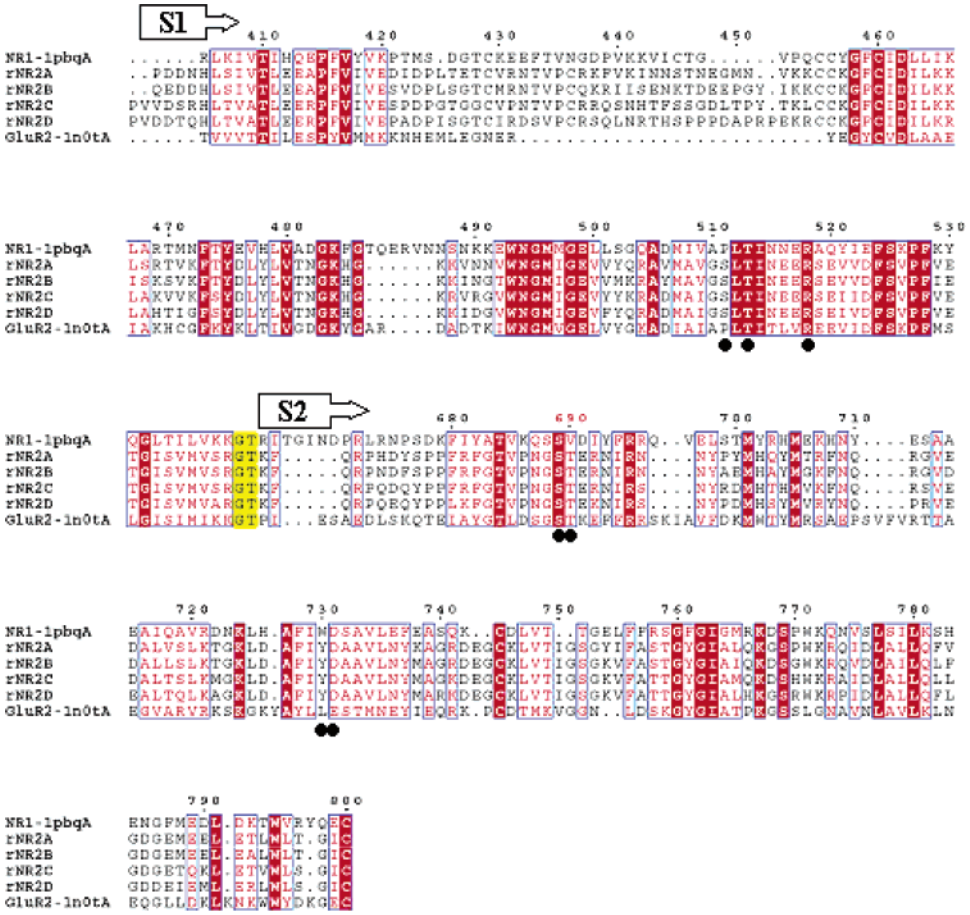

Figure 2. Sequence alignment. Colors are chosen according to the following rules: red box and white characters represent strict identity. Red characters represent similarity in a group, and blue frames represent similarity across groups. Numbering is that of the NR2A sequence, and the yellow box (GT) represents the linker GT.[20] Filled circles below the sequences indicate the residues involved in ligand binding.

respect to the assessment of the structure−activity relationship, we docked flexible NMDA ligands structurally related to (*R*)-AP5 and checked the amino acids of the binding site involved in the interaction. The results show that the docked ligands assume a binding orientation within the binding site that is in accordance with the results of site-directed mutagenesis.[16,17] The most striking residue is T690A because its replacement produces a 255-fold reduction of the (*R*)-AP-5 binding affinity. The flexible docking of (*R*)-AP-5 into our NR2A model showed that residues S511, T513, H485, R518, G688, S689, T690, and D731 are involved in the interaction (Figure 4). Worth noting is that the similar residues at the same position play a critical role in the binding site of the AMPA-GluR2 receptors (Figure 3).

The side chain of the highly conserved R518 is the primary anchor point for the α-carboxyl group of the ligands, whose interaction is further stabilized by hydrogen bonds with T513. The α-amino group of (*R*)-AP5 is lodged in the negative charged pocket formed by the carbonyl group of the backbone of S511, the hydroxyl group of T513, and the carboxylate of D731. The docking confirms the interaction of the *ω*-acidic group with the amino terminal portion of the α-helix, corresponding to the α-helix F of the GluR2 template. If these conclusions could be extended to all NR2/NMDA subunits, this outcome is in contrast with the results published for NR2B/S1−S2 by Tikhonova et al.[26] and Laube et al.[27] who showed that the phosphonic group

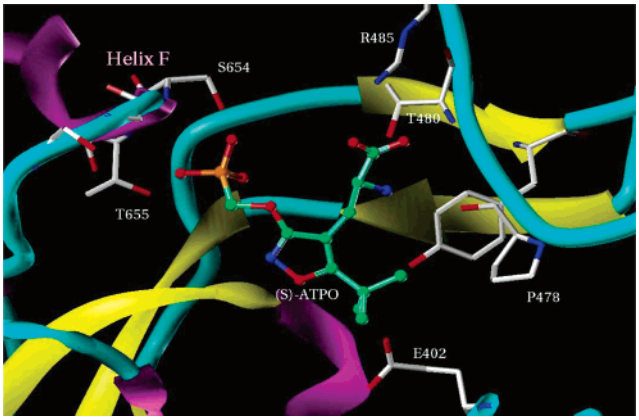

Figure 3. Binding mode of (*S*)-ATPO cocrystallized into the GluR2 subunit. The ligand (green) is displayed as ball-and-stick model, while the amino acids involved in ligand binding are color-coded as capped sticks.

of (*R*)-AP5 does not interact with residues that are analogues to G688−T690 (G689−T691 in NR2B) but with a positive charged pocket located around K485−K488 (K484 and K487 in NR2A) and the backbone of S690 of NR2B. The authors stated that antagonists dock mainly to the S1 surface occupying the binding site of the glutamate amino acidic group, thereby preventing the channel activation. We believe that in the NR2A subunit the phosphonic group, and in general the *ω*-acidic group, interacts directly with the region close

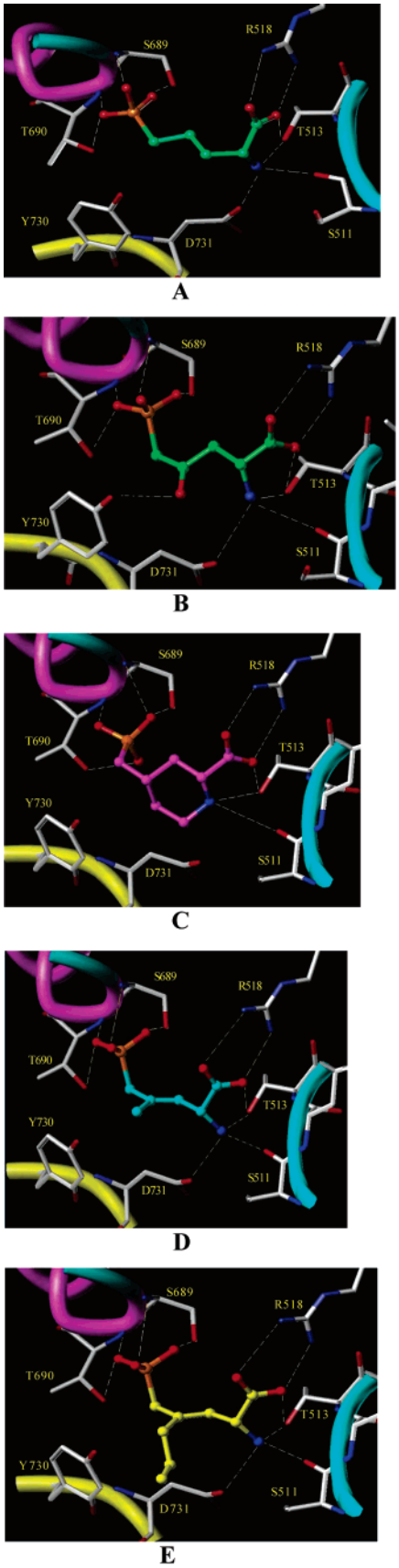

Figure 4. Docking of the antagonists in Table 1 into the new model of the NR2A subunit: (A) (*R*)-AP5; (B) MDL 100,453; (C) CGS 19755; (D) CGP 37849; (E) CGP 39653. The ligands are displayed in a ball-and-stick model, while the amino acids involved in ligand binding are color-coded as capped sticks. H-bonds are shown as gray dotted lines.

Table 1. Apparent Affinities (IC_{50}) and GOLD Scores of (*R*)-AP5-like NMDA Antagonists[a]

ligand	IC_{50} (μM) [^{3}H]CPP	IC_{50} (μM) [^{3}H]Glu	GOLD score
(*R*)-AP-5	0.29 ± 0.048	1.5 ± 0.2	74.62
MDL 100,453	0.11 ± 0.01	nd[b]	76.46
CGS 19755	0.095 ± 0.028	0.84 ± 0.39	72.32
CGP 37849	0.035 ± 0.020	0.22 ± 0.04	76.26
CGP 39653	nd[b]	0.23 ± 0.04	77.44

[a] Data taken from ref 1. [b] nd: not determined.

to G688−T690, that is, the N-terminal portion of a α-helix. It is important to stress that α-helices generate a macro dipole moment, whose magnitude corresponds to 0.5−0.7 unit positive charge at the N-terminal end of the helix, which is known to be favorable for binding phosphate groups.[28] In analogy, the phosphonic group, which characterizes the most potent NMDA antagonists, forms an optimal interaction with the macrodipole and the NH group of the N terminus of the α-helix above-described. This observation finds support in the higher affinity of the phosphono derivative (*R*)-AP5 in comparison to (*R*)-aminoadipic acid, the corresponding carboxylate counterpart. It is interesting to note that residue K484 (NR2A) is highly conserved in all iGluR sequences and, as evidenced by the published crystal structures of GluR2,[14] GluR6,[29] and NR1,[15] is involved in the interdomain contacts. The same role is reasonably played by amino acid K487, which is located on lobe I and is highly conserved in the NR2 sequence. As a consequence, the replacement of residue K484 decreases the affinity of endogenous agonists glutamate at NR2A,B/GluR2 subunits and glycine at the NR1 subunit.[7] The recently reported X-ray structure of the GluR6 binding domain cocrystallized with domoic acid[29] (DA, Figure 1) reveals that the receptor activation by this agonist is due to its amino acidic moiety and the γ-carboxylate group. In this binding orientation the more remote carboxylate of domoate (ω-COOH) interacts with the NH of Y488 located on lobe I without any apparent contact with lobe II. This X-ray structure shows that the moiety of the binding site that was suggested to be occupied by the antagonists[26,27] can be taken by the remote carboxylate of an agonist.

The flexible docking calculations of the AP5-like NMDA selective antagonists listed in Table 1 clearly show a consensus in their binding mode (Figure 4). Furthermore, the scoring of the docking program for these ligand poses (GOLD score in the range 72−77) is in agreement with their apparent affinities. However, since the experimentally determined values are within 1 order of magnitude, the docking methodology is not able to discriminate the ligands because of the intrinsic limit in its accuracy.[30]

At variance with the model derived from the GluR2/S1−S2 template,[14] docking results derived from the model built on the NR1 template were not in accordance with the previously discussed mutagenesis data.[16,17] We believe that the main reason for such a discrepancy resides in the different sequences of the NR1 and NR2A active sites, especially in the string SSV (687−689) of NR1, whose amino acids are replaced by GST (688−690) in NR2A. In addition, despite the high identity with our target, the NR1 protein endogenously binds the less bulky glycine compared to glutamate. As a consequence,

models built on this template could be inadequate to account for the docking of NMDA ligands other than glycine.

In summary, this study confirms literature data indicating that the distance between S1 and S2 domains is the critical determinant of the receptor for binding the ligands. The GluR2/S1−S2-based model allowed relating of the docking results to the pharmacological data of the compounds under investigation. In contrast, the model derived from the NR1/S1−S2 template was not informative because the amino acid residues involved in the ligand binding were too close to account for the data collected on AP5-like NMDA antagonists. Moreover, at variance with the results reported in the models of the NR2B subunit built only on the GluR2/S1−S2 (DNQX) template by Tikhonova[26] and Laube,[27] we are suggesting a different binding mode for the NMDA antagonists at the NR2A/S1−S2 construct. We propose that the binding of AP5-like NMDA antagonists at the NR2A receptor is quite similar to that of ATPO at the GluR2 AMPA receptor. However, both of the proposed binding modes are consistent with the results of the site-directed mutational analysis reported for the NR2A and NR2B subunits. On the other hand, the bioactive conformations of the reported AP5-like ligands are consistent with the known pharmacophore model.[31] In particular, CGS 19755 adopts a "folded" conformation with the negative charged groups located on the same side of the molecule (syn) as reported by Bigge et al.[31] We believe that the predicted AP5 binding mode can be extended to ligands with a carbon chain longer than AP5, i.e., AP7 or its aryl analogues. We are confident that the NR2A model proposed in this work could be helpful for understanding the pharmacological data of subunit selective ligands reported in the literature and for designing new NR2A subunit selective ligands.

Methods and Experimental Section

Comparative Modeling. All the sequences were acquired from the ExPASy molecular biology server.[32] The multiple alignment was performed utilizing MALIGN implemented in the BODIL modeling environment,[33] and the scores were obtained using the Dayhoff matrix.[34] The 3D model of the target protein was built by means of the "first approach mode" of the SWISS-MODEL server (SMS).[35−37] This very functional computational tool automatically aligns the sequences by BLAST,[38] builds the crude 3D model, and then minimizes the structure using GROMOS96[39] performing 200 cycles by the steepest descent method and 300 cycles by the conjugate gradient method.

NR2A Model Built on GluR2. Checking the sequence of the model obtained by SMS, we noted that the structure began from the 46th residue of the submitted sequence. To skip this difficulty, we submitted only the first 70 amino acid residues of the entire target sequence to SMS and used GluR2/S1−S2 in complex with ATPO (PDB code 1n0t) and NR1/S1−S2 in complex with DCKA (PDB code 1pbq) as templates. The conformation of the 24 amino acid segment missing in the AMPA subunit, corresponding to the amino acid string 432−455 in the NR2A sequence, could be predicted by means of the available crystallized NR1 subunits. We joined the two components of the model, superimposing the first common α-helix and pasting the missing part to the first component. The side chains of the nonidentical amino acids were modeled using the Biopolymer module implemented in the Sybyl 6.8[40] molecular package running on an SGI workstation. The conformations of the nonconserved residues side chains were visually inspected, and their torsion angles were fixed by considering the values of the amino acidic residues of both templates. Later, the protein containing (*R*)-AP5 was solvated with a cubic box of water (TIP3P model) and minimized by the AMBER 7.0[41] suite program. The Parm99 force field was used for high-quality minimization. In the first step, only atoms of water and the side chains of the protein were allowed to move while the rest of the molecule was kept frozen. Next, the entire system was minimized. The model refinement was done by a molecular dynamics simulation with the SHAKE approximation implemented in AMBER. Initially, to equilibrate the system, keeping the volume constant, only the water molecules were heated from 0 to 300 K in a 20 ps step. Successively, during a simulation of 20 ps, the entire system was equilibrated heating the protein from 50 to 300 K. Finally, the trajectories were computed at a temperature of 300 K controlled by the Berendsen's algorithm.[42] The time step was set to 2 fs, and the energy and the coordinates information were printed every 250 steps. The dynamics simulation was performed with anisotropic pressure scaling for a period of 1 ns. After this time no change in the (*R*)-AP5 binding mode was noticed. The stereochemical quality of the protein models was checked using the Procheck3.0[20,21] program with a pseudoresolution of 1.5 Å.

NR2A Model Built on NR1. This model was built using the same strategy as described before. In this case, the available crystallized open form of the NR1 subunits (PDB code 1pbq) was used as the template.

Ligand Minimization. All ligands were built using Sybyl,[40] and they were preliminarily minimized by the Tripos force field and then reoptimized at the MOPAC/PM3 level. To simulate the physiological conditions better, the amino, carboxyl, and phosphonate groups were used in their charged forms. The charges were assigned with the Gesteiger−Huckel method.

Molecular Docking of the Ligand. We used as a putative binding site a region of the NR2A protein surrounding the amino acids that have been described as essential for L-Glu and (*R*)-AP5 binding.[16,17] GOLD 2.2,[43,44] used for the molecular docking calculations, employs a genetic algorithm (GA) wherein the molecular features of the protein/ligand complexes are encoded as a chromosome. The protein required for the calculations was considered with all hydrogen atoms, and the implemented algorithm successively optimized the orientation of the hydroxyl hydrogen atom of residues Tyr, Thr, Ser, and Lys located in the binding pocket. Lone pairs were automatically added with the default geometry. The cavity was detected with an active site radius of 12.0 Å from the side chain hydrogen atom of residue T513. The GOLD score fitness function and the torsion angle distributions were chosen to improve the quality of the docking results. van der Waals and hydrogen-bonding radii were set to 4.0 and 3.0 Å, respectively; the genetic algorithm parameters were kept at the default value. Finally, the obtained docking results were visually analyzed by Sybyl to evaluate the quality and the accordance of the resulting binding mode with the experimental data.

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