

Structural Basis of NR2B-Selective Antagonist Recognition by N-Methyl-D-aspartate Receptors^[S]

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ABSTRACT

N-Methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors endowed with unique pharmacological and functional properties. In particular, their high permeability to calcium ions confers on NMDARs a central role in triggering long term changes in synaptic strength. Under excitotoxic pathological conditions, such as those occurring during brain trauma, stroke, or Parkinson's or Huntington's diseases, calcium influx through NMDAR channels can also lead to neuronal injury. This argues for the use of NMDAR antagonists as potential therapeutic agents. To date, the most promising NMDAR antagonists are ifenprodil and derivatives, compounds that act as noncompetitive inhibitors selective for NMDARs containing the NR2B subunit. Recent studies have identified the large N-terminal domain (NTD) of NR2B as the region controlling ifenprodil sensitivity of NMDARs. We present

here a detailed characterization of the ifenprodil binding site using both experimental and computational approaches. 3D homology modeling reveals that ifenprodil fits well in a closed cleft conformation of the NRB NTD; however, ifenprodil can adopt either of two possible binding orientations of opposite direction. By studying the effects of cleft mutations, we show that only the orientation in which the phenyl moiety points deep toward the NTD hinge is functionally relevant. Moreover, based on our model, we identify novel NTD NR2B residues that are crucial for conferring ifenprodil sensitivity and provide functional evidence that these residues directly interact with the ifenprodil molecule. This work provides a general insight into the origin of the subunit-selectivity of NMDAR noncompetitive antagonists and offer clues for the discovery of novel NR2B-selective antagonists.

NMDA receptors (NMDARs) are glutamate-gated ion channels widely expressed in the central nervous system that mediate a component of excitatory synaptic transmission. NMDARs are essential for normal physiological processes, such as brain development, synaptic plasticity, learning, and memory (Dingledine et al., 1999). NMDARs are also involved in many brain disorders, triggering an intense interest as potential therapeutic targets. They are best known for their role in excitotoxicity, a process during which excessive glu-

tamate release causes overactivation of NMDARs, accumulation of intracellular calcium and, eventually, neuronal death. Excitotoxicity occurs during many acute (brain trauma, stroke) and chronic neurodegenerative disorders (Alzheimer's, Parkinson's, Huntington's diseases). Overactivity of NMDARs is also observed in epilepsy and chronic pain (Kemp and McKernan, 2002). To counteract the deleterious effects of NMDAR overactivation, extensive efforts have been made to discover potent and selective NMDAR antagonists. In the 1980s, the first compounds to be developed were competitive antagonists and high-affinity channel blockers. However, despite good efficacy against neuronal injury, most of these early NMDAR antagonists failed in clinical trials because of unacceptable side effects including hallucinations, drowsiness, memory, and motor deficits (Kemp and McKernan, 2002). One likely explanation for the failure of these first-generation NMDAR antagonists is their lack of subunit specificity. By targeting the agonist-binding domain (competitive antagonists) or the

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ABBREVIATIONS: NMDAR, N-methyl-D-aspartate receptor; NMDA, N-methyl-D-aspartate; NTD, N-terminal domain; LIVBP, leucine/isoleucine/valine-binding protein; 3D, three-dimensional; wt, wild type; ABD, agonist-binding domain.

ion pore (channel blockers), these compounds do not discriminate between the various NMDAR subtypes and cause generalized inhibition of NMDAR activity.

In vivo, NMDARs occur as multiple subtypes most often composed of NR1 and NR2 subunits. They form heterotetrameric complexes made of two NR1 and two NR2 subunits. Although NR1 is encoded by a single gene, the NR2 subunit exists as four subtypes encoded by four different genes (NR2A-D), each with a distinctive spatiotemporal pattern of expression. Different subunit composition imparts different biophysical and pharmacological properties (Cull-Candy and Leszkiewicz, 2004; Paoletti and Neyton, 2007). One of the most exciting recent developments in NMDAR pharmacology has been the identification of highly subtype-selective antagonists that act allosterically (in a noncompetitive manner). As a matter of fact, these agents are much better tolerated compared with broad-spectrum NMDAR antagonists (Kemp and McKernan, 2002). The most promising subtype selective compounds are ifenprodil and derivatives, a large family of synthetic compounds that selectively inhibit NMDARs containing the NR2B subunit (Williams, 1993; Mott et al., 1998; Hatton and Paoletti, 2005). Among them, several highly potent molecules show good efficacy as neuroprotectants and/or painkillers in a variety of animal models (Chizh et al., 2001; Chazot, 2004; Gogas, 2006). It is noteworthy that in humans, NR2B-selective antagonists do not induce the adverse side effects usually seen with nonselective NMDAR antagonists, even at maximally neuroprotective doses (Chizh et al., 2001; Gogas, 2006). Despite these encouraging data, NR2B-selective antagonists have not succeeded in clinical trials yet because of poor oral bioavailability and pharmacokinetic profiles (Kew and Kemp, 2005). Thus, new potent NR2B-selective antagonists are still in great demand.

The ifenprodil binding site on NMDARs has been mapped to the N-terminal domain (NTD) of the NR2B subunit (Gallagher et al., 1996; Perin-Dureau et al., 2002; Malherbe et al., 2003). The NTD, composed of the first ~380 amino acids, is present in all eukaryotic ionotropic glutamate receptor subunits and participates in subunit assembly (Mayer, 2006). In NR2A and NR2B subunits, the NTD also forms a modulatory domain binding the Zn^{2+} ion, which acts as an endogenous allosteric inhibitor of NMDARs (Choi and Lip-ton, 1999; Low et al., 2000; Paoletti et al., 2000; Rachline et al., 2005; Gielen et al., 2008). NR2A and NR2B NTDs form discrete modules because they are still capable of binding zinc or ifenprodil when produced in isolation from the remainder of the receptor complex (Perin-Dureau et al., 2002; Rachline et al., 2005; Wong et al., 2005). NMDAR NTDs share weak sequence similarity with some bacterial periplasmic binding proteins like LIVBP (leucine/isoleucine/valine-binding protein; Masuko et al., 1999; Paoletti et al., 2000). Their structure has not been determined yet, but they are thought to fold as two lobes separated by a hinge, similarly to LIVBP. Using a mutagenesis approach, Perin-Dureau et al. (2002) suggested that ifenprodil binds in the central interlobe cleft of NR2B NTD and promotes cleft closure through a hinge-bending motion (Venus Flytrap mechanism). However, despite the plentiful production of ifenprodil-derived NR2B-selective antagonists (Chenard and Menniti, 1999; Nikam and Meltzer, 2002), the binding mode of ifenprodil and its derivatives on NR2B NTD remains ill defined. It is unclear which of the NTD residues directly interact with the ligand

and what is the structural basis for the subtype-selective pharmacology conferred by the NTDs. Marinelli et al. (2007) proposed a model of ifenprodil binding into NR2B NTD; because no experimental validation was performed, however, it remains a theoretical proposal. In this study, we combine molecular modeling and functional approaches to provide a realistic 3D model of the NR2B NTD-ifenprodil complex.

Materials and Methods

Molecular Biology

The pcDNA3-based expression plasmids for rat NR1-1a (named NR1 herein), rat NR2A, and mouse $\epsilon 2$ (named NR2B herein), the mutagenesis strategy, the sequencing and the RNA synthesis have been described previously (Paoletti et al., 1997, 2000; Rachline et al., 2005).

Electrophysiology

Recombinant NMDA receptors were expressed in *Xenopus laevis* oocytes after coinjection of 30 nl of a mixture of cDNAs (10 ng/ μ l; nuclear injection) or cRNAs (10–100 ng/ μ l) coding for wild-type NR1-a and various NR2B subunits (ratio 1:1). Oocytes were prepared, injected, voltage-clamped and superfused as described previously (Paoletti et al., 1997). The standard external solution contained 100 mM NaCl, 0.3 mM $BaCl_2$, 5 mM HEPES, and 2.5 mM KOH. The pH was adjusted to 7.3 with HCl. NMDA currents were induced by simultaneous application of saturating concentrations of L-glutamate and glycine (100 μ M each) and recorded at -60 mV. Zinc solutions were obtained by diluting in the agonist solution a 100 mM $ZnCl_2$ stock-solution prepared in 0.1 N HCl. In these solutions, zinc was not buffered, and the control zinc-free solution was made by adding 10 μ M diethylenetriamine-pentaacetic acid to chelate trace amounts of contaminant zinc (Paoletti et al., 1997). Ifenprodil was prepared as 100- μ l aliquots (in bidistilled water) at 10 mM and stored at $-20^\circ C$. For pH sensitivity experiments, solutions were prepared according to Gielen et al. (2008). When performing pH dose-response curves, at very alkaline pH values, glutamate and glycine concentrations were adjusted to compensate for the loss of protonation of the α -amino moiety ($pK_a \sim 9.7$ for both L-glutamate and glycine). Thus, for pH of 9.3 and 10.3, glutamate and glycine concentrations were increased by 1.4- and 5-fold, respectively. All experiments were performed at room temperature.

Data Analysis

Data were collected and analyzed using pClamp 9.2 (Molecular Devices, Sunnyvale, CA). They were fitted using Sigmaplot 8.0 (SSPS, Chicago, IL). For ifenprodil dose-response curves, experimental points were fitted using the following Hill equation: $I_{ifen}/I_{control} = 1 - a/(1 + (IC_{50}/[ifen])^{n_H})$, where $I_{ifen}/I_{control}$ is the mean relative current, [ifen] is the ifenprodil concentration, IC_{50} is the concentration of ifenprodil producing 50% of the maximal inhibition, n_H is the Hill coefficient, and a is the maximal inhibition at the saturating ifenprodil concentration. IC_{50} , a , and n_H were set as free parameters. Zinc dose-response curves were fitted using the following Hill-derived equation: $I_{zinc}/I_{control} = 1 - a/(1 + (IC_{50}/([Zn] + b))^{n_H})$, where b is the contaminant zinc concentration and [Zn] the added zinc concentration. Following Rachline et al. (2005), b was set to 100 nM. pH dose-response curves were analyzed according to Gielen et al. (2008). Error bars represent the standard deviation of the mean of relative currents.

Cysteine Affinity Labeling

N-[4-[2-(4-Benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide (molecule 4 from Alarcon et al., 2008) was prepared at 500 mM in anhydrous dimethyl sulfoxide. Initial agonist response (I_0) was measured in oocytes. Then, each oocyte was removed from the re-

cording chamber and incubated in a 100 μl of Barth solution [88 mM NaCl, 1 mM KCl, 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , 2.4 mM NaHCO_3 , and 10 mM HEPES; pH adjusted to 7.6 with NaOH] containing gentamicin (50 $\mu\text{g}/\mu\text{l}$), glutamate (100 μM), 5,7-dichlorokynurenic acid (40 μM), and a 500 μM concentration of the reactive ifenprodil derivative (*N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide). After a 30-min incubation at room temperature, oocytes were washed for 1 min in a Barth solution containing gentamicin (50 $\mu\text{g}/\mu\text{l}$). We verified that, on wild-type (wt) NR1/NR2B receptors, 1 min of wash-out was sufficient for a near recovery of the initial agonist-induced response after an application of 500 μM *N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide. Then agonist response was measured again (I_{incub}). The ratio I_{incub}/I_0 is reported for each oocyte. Error bars represent the standard error of the mean ratio I_{incub}/I_0 .

In preliminary control experiments, we observed that incubation in 500 μM *N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide induced a decrease of the NMDA response carried by wt NR1/NR2B receptors ($I_{\text{incub}}/I_0 = 0.35 \pm 0.06$; $n = 6$). An endogenous cysteine, C232, is located in the vicinity of the ifenprodil binding-site of NR2B and could possibly react with *N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide. However, a similar I_{incub}/I_0 ratio was found for NR1wt/NR2B-C232A receptors, showing that the irreversible inhibition seen on wt receptors was not due to irreversible labeling of this NTD cleft cysteine. This hypothesis was confirmed by incubating oocytes expressing wt NR1/NR2B receptors in a 100 μM concentration of the nonreactive ifenprodil molecule, which also yielded a strong decrease in the NMDA response ($I_{\text{incub}}/I_0 = 0.10 \pm 0.04$; $n = 10$) [In this experiment, because of the slow wash-out of ifenprodil inhibition (Perin-Dureau et al., 2002), oocytes were washed for 10 min after incubation in ifenprodil.] In contrast, incubation in 100 μM ifenprodil induced very little irreversible inhibition of receptors truncated for the entire NR2B NTD (NR1wt/NR2B- Δ NTD) ($I_{\text{incub}}/I_0 = 0.9 \pm 0.1$; $n = 5$) or of receptors containing a single strong ifenprodil binding mutation (NR1/NR2B-D101A or NR1/NR2B-V262D receptors; data not shown). Therefore, at high concentrations, ifenprodil and derivatives can induce an NTD-dependent irreversible inhibition of NR2B-containing NMDARs without involving any covalent binding of these molecules. The magnitude of this noncovalent irreversible effect was found to depend on the affinity of the receptor for the ifenprodil derivative (data not shown).

Molecular Modeling Studies

Homology Modeling of NR2B NTD. A sequence alignment of rat NR2A and NR2B NTDs with the agonist-binding domain of mGluR1 was generated according to Malherbe et al. (2003) and further refined using predicted (NR2A and NR2B) and known (mGluR1 agonist-binding domain; PDB code 1ewk:A) secondary structures. Secondary structure elements of NR2A and NR2B NTDs were predicted using PROF predictions (<http://www.predictprotein.org>; Rost and Sander, 1993). Homology models for NR2B NTD were generated by the automated comparative modeling tool MODELER 9.0 (DS Modeling 1.7; Accelrys, San Diego, CA) as described previously (Bertrand et al., 2002). Models were generated by using the coordinates of the mGluR1 agonist-binding domain closed form (PDB code 1ewk:A) and based on the sequence alignment described in Fig. 1. The structural quality of the models was assessed according to the MODELER probability density functions as well as Profiles-3D analysis (DS Modeling 1.7). Loops were refined using MODELER. The final selected model was used for docking.

Docking of Ifenprodil in the Model of NR2B NTD. Ifenprodil was docked using LigandFit (Venkatchalam et al., 2003) (DS Modeling 1.7). In such a process, the protein is kept rigid while the ligands undergo Monte Carlo conformational searching. Twenty poses were generated, clustered, and selected according to their binding mode.

Docking Refinement of Ifenprodil in NR2B NTD. The obtained protein–ligand complexes were submitted to energy minimi-

zation while tethering the $\text{C}\alpha$ trace. This was performed using the CHARMM calculation engine (Brooks et al., 1983; DS Modeling version 1.7). CHARMM was also used to perform 1 ns of molecular dynamics at 298 K. Once the system was equilibrated, snapshots were collected, averaged, and submitted again to energy minimization (Bertrand et al., 2002).

Qualitative Pharmacophore Models Generation. Five molecules that had the same activity as ifenprodil were selected. The pharmacophore models were generated by using the qualitative common feature pharmacophore HipHop algorithm (Barnum et al., 1996) of Catalyst 4.11 (Accelrys).

Results

Molecular Modeling of the N-Terminal Domain of NR2B and Docking of Ifenprodil. We built a 3D model of the NTD of NR2B using the atomic coordinates of the closed agonist-binding domain (ABD) of the metabotropic glutamate receptor mGluR1 as a template (PDB code 1EWK:A; Kunitz et al., 2000). mGluR1 ABD is indeed another LIVBP-like protein that shares slightly more sequence identity with NR2B NTD than LIVBP does [$\sim 12\%$ identity for rat NR2B NTD (protein ID Q00960.1)/rat mGluR1 ABD (protein ID P23385.1) versus $\sim 9\%$ for NR2B NTD/LIVBP (PDB code 2liv_A)]. 3D models were generated using the alignment shown in Fig. 1. Because the sequence identity between the NR2B NTD and mGluR1 ABD is very low ($\sim 12\%$), we based our alignment according to known (mGluR1 ABD) and predicted (NR2B NTD) secondary structure elements (Fig. 1). In the loops putatively lining the central cleft of NR2B NTD (Perin-Dureau et al., 2002; Rachline et al., 2005), we also stipulated that residues previously shown to control zinc and/or ifenprodil inhibition should be aligned with residues pointing toward the glutamate binding cleft of mGluR1 ABD. This is obviously a strong constraint. Consequently, we took special care in our subsequent functional experiments to assay the role of these residues pointing toward the NR2B NTD cleft.

The structural quality of the generated 3D models was assessed according to the MODELER probability density functions (PDF energy), as well as Profiles-3D analysis (P3D score). The structural quality of the models was further improved by individually refining loops lining the binding cleft. The final NR2B NTD model showed a P3D score of 151.66 over 166.35 (91% of the maximal expected score) and exhibited no misfolded region around the binding cleft, attesting to the overall goodness of the chosen model. We also verified that, in addition to its high score, this model showed a good concordance to the imposed constraint (residues controlling zinc and/or ifenprodil sensitivity pointing toward the interlobe cleft of the NTD). This was indeed the case (Fig. 2). However, because the sequences of the NR2B NTD and mGluR1 ABD share such a low sequence identity, the orientations of residues side-chains are rather imprecise. Thorough functional validation of this model is therefore mandatory to confirm its biological relevance.

We then docked ifenprodil in erythro configuration, the synthesis of ifenprodil being diastereoselective (Avenet et al., 1996), into the interlobe cleft of the modeled NR2B NTD, using LigandFit (see *Materials and Methods*). Docking experiments did not reveal a unique binding mode for ifenprodil. Rather, we found that ifenprodil could bind to the NTD of NR2B with two opposite orientations: with its phenol group

close to the entrance of the cleft and its benzyl group contacting the interlobe hinge (orientation 1) or vice versa (orientation 2). Each orientation was further refined by 1 ns of molecular dynamics (see *Materials and Methods*), to yield the two models shown in Fig. 2 (Fig. 2A with orientation 1; Fig. 2B with orientation 2). In both models, ifenprodil binds in extended conformations that correspond to low-energy conformations in the solvent. Moreover, the ifenprodil molecule makes interactions with both lobes.

We next sought to verify that the conformations of ifenprodil found after molecular dynamics in both orientations were likely to be bioactive conformations. Indeed, ifenprodil is a flexible molecule and the conformation with which it actually binds to the NTD is unclear. The bioactive conformation of a ligand can be predicted by the use of qualitative common feature pharmacophore models (Barnum et al., 1996). A common feature pharmacophore model describes the 3D arrangement of the shared electronic properties required

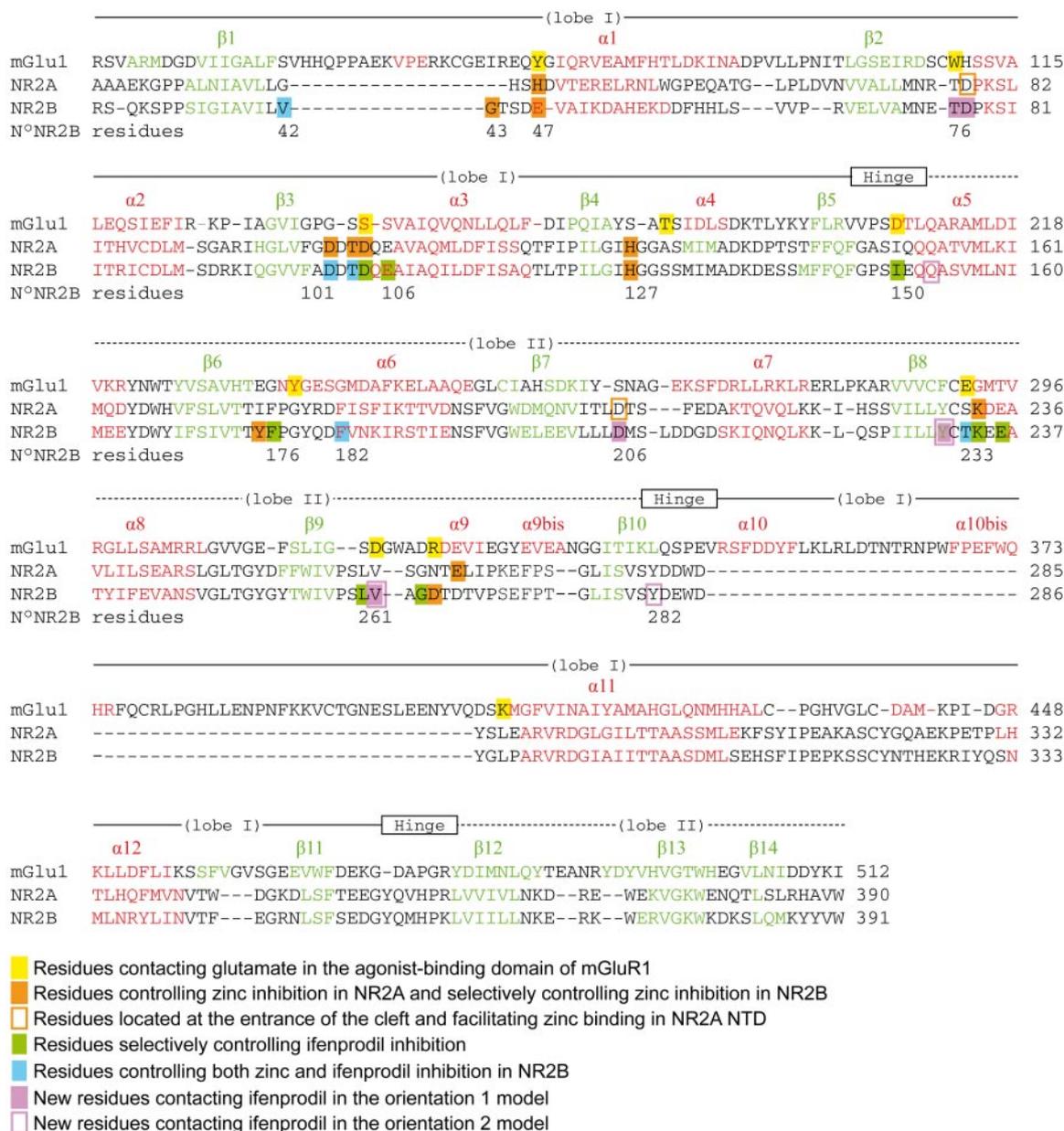
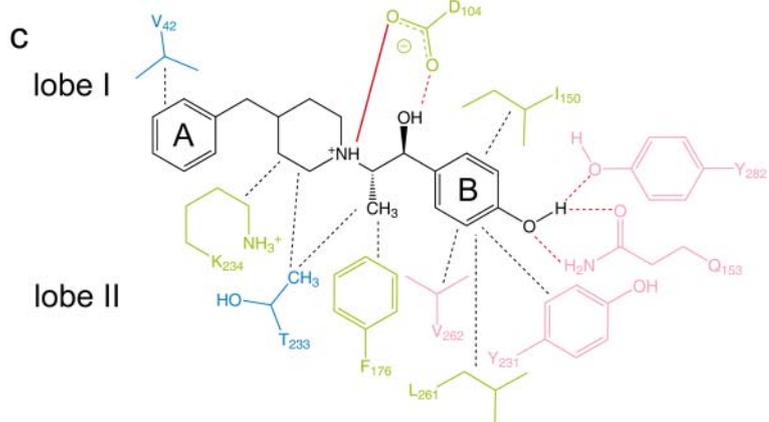
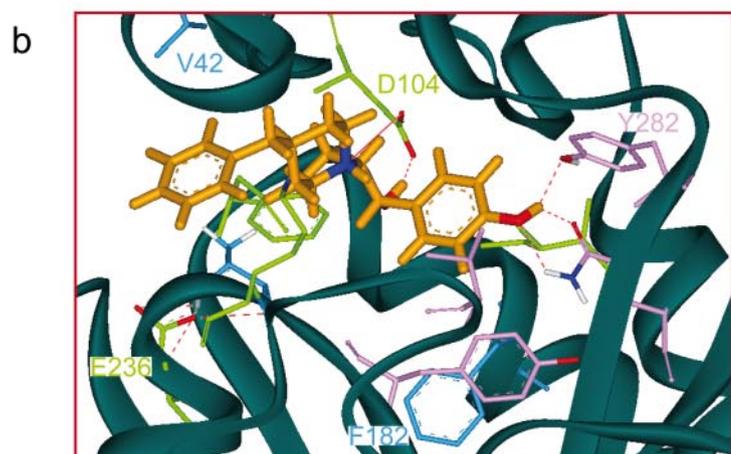
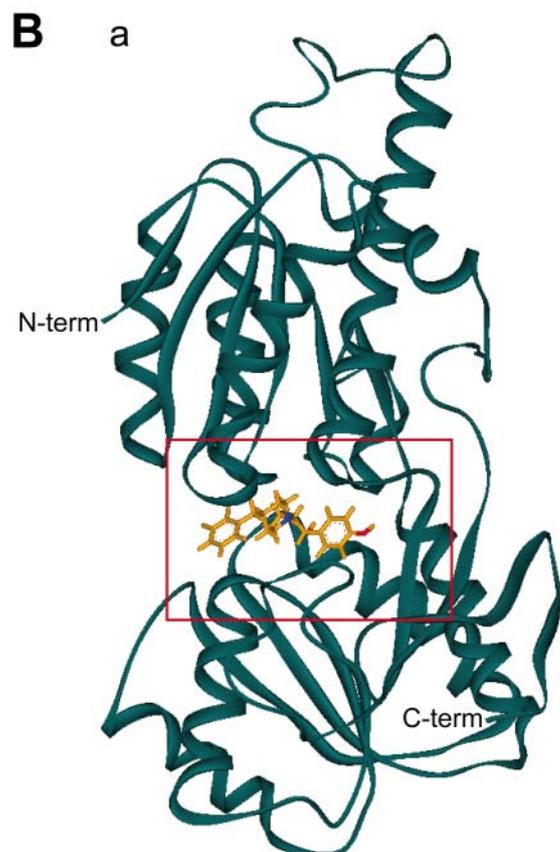
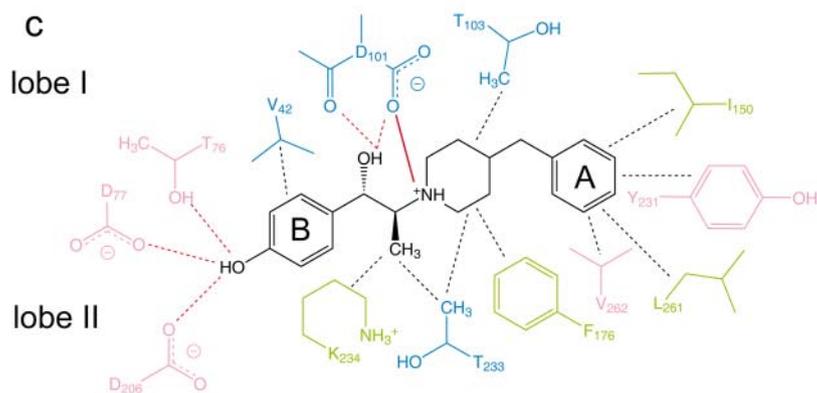
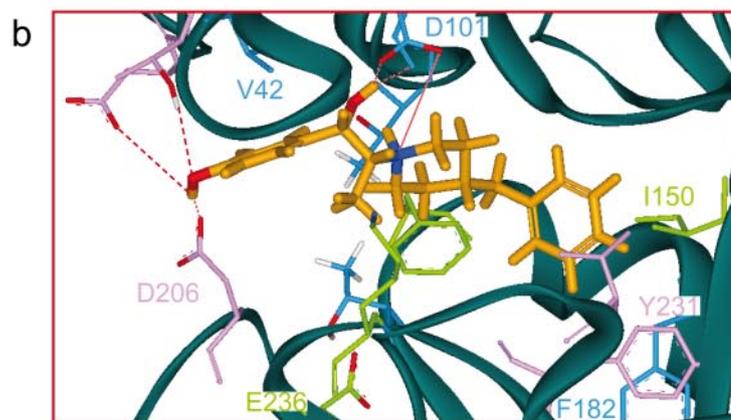
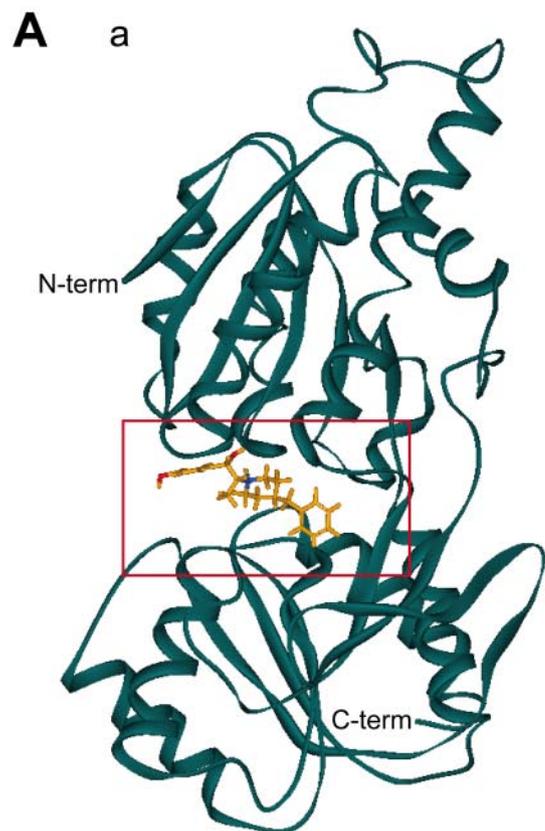


Fig. 1. Sequence alignment of NR2A and NR2B NTDs with the agonist-binding domain of mGluR1. The indicated α -helices (red) and β -strands (green) of mGluR1 are from the X-ray structure of the agonist-binding domain of mGluR1 (PDB 1ewk:A) (Kunishima et al., 2000). Secondary structure elements of NR2A and NR2B NTDs were predicted using PROF predictions (see *Materials and Methods*). Yellow closed boxes correspond to residues of mGluR1 contacting the glutamate molecule in mGluR1 ABD X-ray structure (Kunishima et al., 2000), orange closed boxes to residues of NR2A controlling high-affinity zinc inhibition and to residues of NR2B controlling zinc inhibition and not ifenprodil inhibition (Paoletti et al., 2000 and Rachline et al., 2005), green closed boxes to residues of NR2B selectively controlling ifenprodil inhibition (Perin-Dureau et al., 2002), blue closed boxes to residues of NR2B controlling both ifenprodil and zinc inhibition (Rachline et al., 2005). Mutations into alanine of these highlighted NR2A and NR2B residues all result in a decrease of zinc and/or ifenprodil sensitivity, except for Val42, which decreases ifenprodil sensitivity but increases zinc sensitivity (see Rachline et al., 2005). In NR2A, residues located at the entrance of the NTD central cleft, proposed as residues providing a favorable electrostatic environment for zinc to access the cleft (Paoletti et al., 2000), are depicted in orange open boxes. In NR2B, newly identified residues contacting ifenprodil in the docking models (see *Results*) are depicted in pink closed boxes (orientation 1) and pink open boxes (orientation 2). Note that despite the conserved pattern of secondary structure elements (alternation of β -strands and α -helices), the sequence identity between mGluR1 ABD and NR2B (or NR2A) NTD is very low (~12%).



for the activity of a set of molecules on their receptor. To build the pharmacophore model of ifenprodil-like molecules, we used a dataset of five molecules having similar activities to ifenprodil on wt NR1/NR2B receptors (see Supplementary Table S1). To obtain a relevant pharmacophore model, we chose rigid molecules with distinct chemical structures. The presence of molecules more rigid than ifenprodil, like those containing an acetylenic linker (second and third molecules of Table S1), is important to limit the number of 3D arrangements of the different pharmacophore features. From this data set, several pharmacophore models were generated, and we selected the pharmacophore with the highest rank. We obtained a four-point pharmacophore composed of two aromatic, one positive ionizable, and one H-bond donor features (Fig. 3A). The pharmacophore model obtained is consistent with the previously published structure-activity relationship data (Tamiz et al., 1998; Chenard and Menniti, 1999; and see *Ifenprodil Interactions in Its Binding Pocket*) and predicts an extended conformation of ifenprodil. The two conformations of ifenprodil found after molecular dynamics (orientations 1 and 2) were then mapped to the pharmacophore model. As shown in Fig. 3, B and C, both conformations closely matched the pharmacophore. The conformations of ifenprodil found in the docking models are therefore likely to be both bioactive conformations. Which of the two orientations of ifenprodil is the actual bioactive one remains to be elucidated.

Ifenprodil Interactions in its Binding Pocket. Investigations of the structure-activity relationships of ifenprodil derivatives have established that there are common important structural features for this family of compounds (Tamiz et al., 1998; Chenard and Menniti, 1999). There are four features: 1) a phenyl moiety (ring A) interacting with a hydrophobic pocket; 2) a positively charged central nitrogen atom that can make ionic or charge-dipole interactions with a H-bond acceptor; 3) a second phenyl group (ring B) coupled with a H-bond donor that can make both hydrophobic and polar interactions; and 4) 10 to 12 Å separating ring A from ring B (linker region). It is noteworthy that the two models described above obey these structural requirements. Moreover, they are overall consistent with the previously published mutagenesis data (Perin-Dureau et al., 2002). Indeed, two of the four residues found to have a strong effect on ifenprodil sensitivity after mutagenesis (Ile150, Phe176; IC_{50} more than 60-fold higher than for wt NR2B-containing receptors) are found to be in direct atomic contact with the ifenprodil molecule, irrespective of the orientation 1 or 2 model (Fig. 2). The third residue, Asp101, also directly inter-

acts with ifenprodil in model 1 but not in model 2, where it is farther apart from the ligand. Finally, the fourth residue, Phe182, is buried in lobe II and cannot contact ifenprodil (Fig. 2). However, this residue has been shown to control sensitivity of NR1/NR2B receptors to both ifenprodil and zinc (Rachline et al., 2005), two NR2B NTD ligands of very different chemical nature, indicating that it might be involved in the global structuring of the NTD. Moreover, Phe182 interacts with Tyr231, a residue that directly interacts with ifenprodil (see below). Thus, in addition to its global structural role, Phe182 may also control ifenprodil binding in an indirect manner, via the correct positioning of Tyr231.

In both orientations, ifenprodil shares common hydrophobic interactions with the receptor. Thus, the aromatic ring close to the hinge of the NR2B NTD is in a hydrophobic pocket composed mostly of Ile150, Tyr231, Leu261, and Val262 side chains (Fig. 2). The direct interaction between Ile150 and ifenprodil fits well with the finding that mutation of Ile150 into a shorter alanine strongly and selectively affects ifenprodil inhibition (inhibition by zinc, the other NR2B NTD ligand, is not affected; Rachline et al., 2005). A similar situation applies for Leu261, a residue that interacts with ifenprodil through its side chain C β . In contrast, mutation Val262A was shown to have only a modest effect on ifenprodil sensitivity (Perin-Dureau et al., 2002), a result that, at first sight, seems difficult to reconcile with our observation that Val262 directly contacts ifenprodil in the models. However, it is possible that the effect of the valine-to-alanine mutation is absent because this mutation does not change the polarity of the residue, and therefore has little impact on ifenprodil binding. Obviously, additional substitutions of Val262 need to be tested to validate this interaction (see below).

Tyr231 is another residue contacting the ifenprodil aromatic group in the hinge region. In fact, in both orientation 1 and 2 models, Tyr231 seems to be engaged in multiple interactions not only with the ligand but also with other residues controlling ifenprodil sensitivity such as Leu261, Ile150, and Phe182 (through π -stacking or Van der Waals interactions). The effects of mutations at NR2B-Tyr231 on ifenprodil sensitivity have not been reported so far. From our models, we expect substitutions at this position to significantly affect ifenprodil inhibition (see below).

At the entrance of the cleft, the aromatic ring of the ifenprodil molecule, in both models, is contacting valine 42, which was also found to exert some control of ifenprodil inhibition (Perin-Dureau et al., 2002). Moreover, the aliphatic chain linking the two aromatic moieties is making Van

Fig. 2. Ifenprodil can bind NR2B NTD with two possible orientations. A, orientation 1. a) 3D model of ifenprodil binding into the NTD of NR2B in orientation 1 with ifenprodil B ring close to the entrance of the cleft and ring A contacting the interlobe hinge (see *Results*). The NTD α -carbon backbone is displayed as a dark green ribbon. Ifenprodil is displayed as sticks (carbons and hydrogens in orange, nitrogen in dark blue and oxygens in red). b) Expanded view of the binding of ifenprodil in orientation 1, showing its interactions with residues of the NTD interlobe cleft. Noncarbon atoms are displayed as follows: hydrogens in white, nitrogens in dark blue, and oxygens in red. Only polar hydrogens and hydrogens of threonine methyl groups are represented. Residues selectively controlling ifenprodil inhibition (Perin-Dureau et al., 2002) are displayed with light green carbon chains and residues controlling both ifenprodil and zinc inhibition (Rachline et al., 2005) are displayed with light blue carbon chains. Residues displayed with pink carbon chains represent newly identified residues contacting ifenprodil in the model, and for which mutagenesis data were lacking. H-bonds are displayed as red dotted lines and coulombian interactions as red plain lines. At the bottom of the figure is displayed Glu236 (green), a residue that, in the model, is important for positioning Thr233 (see *Results*). Note also that Leu261 (located next to Val262), a residue selectively controlling ifenprodil inhibition and contacting ifenprodil in this model, is not represented for clarity reasons. c) Ifenprodil binding-pocket. Schematic two-dimensional view of the interactions of ifenprodil in orientation 1 with residues of NR2B NTD (same color code as in Fig. 2Ab). Van der Waals interactions are displayed as black dotted lines, H-bonds as red dotted lines and coulombian interactions as red plain lines. B, orientation 2. The representation conventions used are the same as in Fig. 2A. a) 3D model of ifenprodil binding into the NTD of NR2B in orientation 2: ring B contacts the interlobe hinge, whereas ring A is close to the entrance of the binding cleft. As in Fig. 2A, Leu261 is not displayed for clarity reasons. b) Expanded view of the binding of ifenprodil in orientation 2, showing its interactions with residues of the NTD interlobe cleft. c) The ifenprodil binding-pocket. Schematic two-dimensional view of the interactions of ifenprodil in orientation 2 with the residues of NR2B NTD.

der Waals contacts with Phe176 and the carbon chain of Lys234, two residues that also selectively control ifenprodil inhibition (Rachline et al., 2005). It is noteworthy that Lys234 makes an ionic interaction with aspartates 265 and 267 (lobe II) and glutamate 47 (lobe I); accordingly, it may neutralize the electrostatic repulsion between these three residues when the domain closes.

The main differences between the two models stand in the polar interactions. Whereas the central positively charged amino group of ifenprodil, in orientation 1, makes a coulombian interaction with Asp101, in orientation 2, it interacts with another aspartate (Asp104). In these models, Asp101 and Asp104 also make a charge-dipole interaction with the linker's hydroxyl group. Mutation of Asp101 into an alanine has been shown to strongly affect ifenprodil sensitivity (Rachline et al., 2005). Model 1 accounts well for this result. However, Asp101 also has a very marked effect on zinc sensitivity (Rachline et al., 2005). This suggests that Asp101 may either be a common key residue for the coordination of both ifenprodil and zinc, or that it may play a more global structural role in the NR2B NTD. On the other hand, the mutation of Asp104 into an alanine has a relatively important effect on ifenprodil inhibition (IC_{50} 22-fold higher than for wt) and no effect on zinc inhibition, making it a potential candidate to directly bind ifenprodil (Rachline et al., 2005). This latter result is consistent with the orientation 2 model, in which Asp104 interacts with the amino group and the linker's hydroxyl group. Orientation 1, in contrast, cannot account for the selective effect of Asp104, because this resi-

due is not pointing toward ifenprodil in the corresponding model. It is important to note, however, that the region containing these two aspartates (loop $\beta 3$ - $\alpha 3$) is very different from the corresponding loop of mGluR1, so that its modeled structure is rather uncertain. Hence, at this stage, there is still doubt concerning which residue interacts with the amino group.

Ifenprodil phenolic group (B ring with H-bond donor) interacts with completely different residues depending on its orientation in the NR2B NTD central cleft. In orientation 1 model, ifenprodil phenolic group makes hydrogen bonds with residues located at the entrance of the cleft: Thr76 and Asp77 from lobe I, and Asp206 from lobe II, thus facilitating the closure of the NTD. On the other hand, the ifenprodil phenolic group with orientation 2 makes hydrogen bonds with residues from the hinge, deep in the cleft: Gln153 and Tyr282. None of these polar residues (Thr76, Asp77, Asp206, Gln153, and Tyr282) have been mutated yet. Knowing the effect of their mutation is expected to help discriminate between the two models.

Overall, the two proposed models seem to fit satisfactorily with the previously published mutagenesis data (Perin-Dureau et al., 2002; Rachline et al., 2005), although doubt remains concerning the residues interacting with the amino group of ifenprodil. The good concordance between the molecular modeling results and the previously published functional data by a first assessment of the validity of the proposed models. However, the role of a few residues is still difficult to interpret, such as Glu106 and Glu236. These

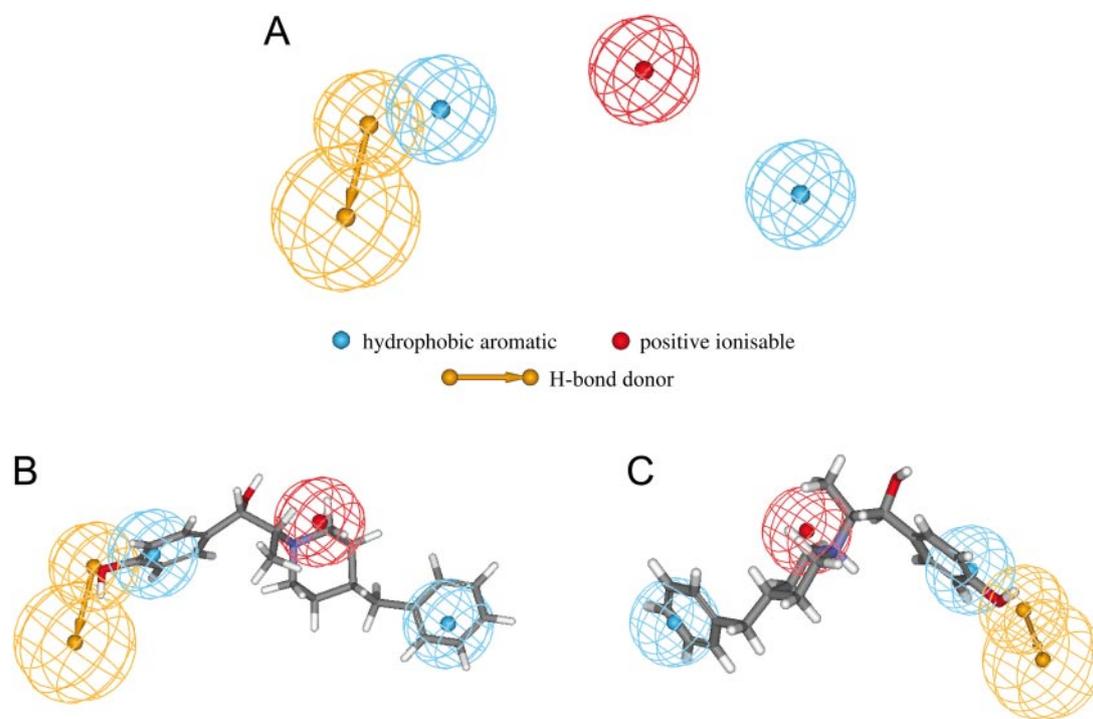


Fig. 3. The conformations adopted by ifenprodil in orientations 1 and 2 are likely to be bioactive conformations. A, pharmacophore model of ifenprodil-like antagonists binding to NR2B NTD. Pharmacophore models are 3D arrangements of electronic features, each feature representing an interaction of the ligand with its receptor. Each feature is symbolized by a central ball representing the application center of the feature in the ligand, surrounded by a tolerance sphere (light blue for hydrophobic features, red for positive ionizable, and orange for hydrogen bond donors). Because H-bonds are directional interactions, the H-bond feature is represented by two balls linked by an arrow that indicates the direction from the heavy atom to the projected point representing the position from which the hydrogen will extend. B and C, mapping of orientation 1 (B) and orientation 2 (C) ifenprodil conformations to the pharmacophore model shown in A. Ifenprodil is represented as sticks, with carbon in gray, hydrogen in white, oxygen in red, and nitrogen in dark blue. Ifenprodil in both orientations 1 and 2 closely fits the pharmacophore model (fit value of 2.34 and 2.85 over 4, respectively).

residues have been shown previously to selectively control ifenprodil inhibition, but they do not contact ifenprodil in both of our models. Glu106 is far from ifenprodil, pointing outside the cleft. Glu236 is buried in lobe II, but its carboxylic group is making hydrogen bonds with the Thr233 hydroxyl group, thus making the methyl group of Thr233 point toward ifenprodil (Fig. 2). Glu236 could therefore act indirectly, via Thr233, Thr233 participating in the control of ifenprodil inhibition (Perin-Dureau et al., 2002). To discriminate between the two orientations of ifenprodil and further attest to the relevance of the chosen model, we used the two complementary functional validation methods developed below.

Asp206, Tyr231 and Val262: Three New Key Residues Controlling Ifenprodil Inhibition. The two models of ifenprodil binding highlighted seven residues that could directly interact with ifenprodil (Asp206, Thr76, Asp77, Gln153, Tyr231, Val262 and Tyr282) but that had not yet been mutated (except for Val262, which had only been mutated into alanine, a rather conservative substitution). If these models are valid, the mutation of these residues with appropriate substitutions should substantially affect ifenprodil inhibition.

The mutation of Val262 into an alanine modestly affects ifenprodil sensitivity of wt NR1/NR2B receptors (Perin-Dureau et al., 2002). As previously mentioned, the lack of effect could be because alanine is still able to make a hydrophobic interaction with the ifenprodil molecule. To verify this hypothesis, we made multiple substitutions of Val262 into either hydrophobic residues of different sizes (glycine, isoleucine, and phenylalanine) or into a weakly polar (cysteine) or a charged (glutamate) residue. Typical current traces for three different mutated and wt NR1/NR2B receptors are shown in Fig. 4A. Whereas substitution of Val262 into an isoleucine slightly increases inhibition by 300 nM ifenprodil [$75 \pm 7\%$ ($n = 5$) versus $63 \pm 6\%$ inhibition for wt ($n = 9$)], mutations V262F or V262D almost completely abolish inhibition by 300 nM ifenprodil [$9 \pm 3\%$ inhibition each ($n = 3-4$)]. Ifenprodil full dose-response curves confirm that mutation of Val262 into a bulky phenylalanine or a charged acidic aspartate strongly decreases ifenprodil sensitivity (50-fold rightward shift in IC_{50}), whereas mutation into an isoleucine has an opposite effect, slightly increasing ifenprodil sensitivity (1.8-fold leftward shift in IC_{50} ; Fig. 4B and Table 1). The very low ifenprodil sensitivity of NR1/NR2B-V262F is likely to be due to the bulkiness of the phenylalanine residue, thus hindering ifenprodil binding to lobe II. Likewise, in NR1/NR2B-V262D receptors, the presence of an aspartate in a hydrophobic pocket is expected to strongly disrupt ifenprodil binding to lobe II by electrostatic repulsion with the negative partial charges of the aromatic ring. In contrast, adding a supplemental methyl group, as occurs with the isoleucine mutation, may increase ifenprodil sensitivity by reinforcing hydrophobic interactions between the NTD and its ligand. We also found that mutation of Val262 into a small weakly polar residue (cysteine) induces only a modest shift in ifenprodil sensitivity (2-fold rightward shift in IC_{50} ; Table 1), comparable with the shift observed with the alanine substitution. Finally, mutation of Val262 into a glycine, which removes any possible side chain interaction with ifenprodil, has a significantly larger effect on ifenprodil sensitivity, increasing ifenprodil IC_{50} 6-fold (Fig. 4B). Contrasting with these side-chain specific effects of Val262 mutations on ifen-

prodil sensitivity, zinc sensitivity was not, or only weakly, affected by any of the Val262 mutations. In particular, mutations NR2B-V262F and NR2B-V262D, which yield the strongest impairments of ifenprodil inhibition, had only little effect on zinc sensitivity (Fig. 4 and Table 1). The differential and selective effects of the various Val262 mutations of ifenprodil sensitivity strongly support a model in which Val262 makes a direct hydrophobic interaction with ifenprodil.

Tyrosine 231 is an additional residue potentially key for ifenprodil binding. In both orientation 1 and orientation 2 models, Tyr231 directly interacts through its aromatic ring with ifenprodil ring A (orientation 1) or B (orientation 2). The aromatic ring of Tyr231 also makes direct contact with the side chains of Phe182, Leu261, and Ile150, the two latter residues also being in direct interaction with ifenprodil. It thus seems that Tyr231 is at the center of a large hydrophobic cluster that forms part of the hydrophobic pocket where ifenprodil ring A (or B) nestles. Accordingly, mutations of Tyr231 are expected to significantly alter ifenprodil sensitivity. This is precisely what we observed. Replacing Tyr231 by a cysteine or an alanine to disrupt the interactions described above resulted in a very strong shift in ifenprodil sensitivity (~ 200 - and ~ 600 -fold shift in IC_{50} , respectively; Fig. 5 and Table 1). These effects are larger than for any other single point mutant studied so far. In fact, the sensitivity of NR1/NR2B-Y231A receptors to ifenprodil is very close to the one of NR1/NR2B- Δ NTD receptors (receptors deleted for the entire NR2B NTD), demonstrating that substituting Tyr231 by an alanine completely disrupts the ifenprodil binding site. In contrast, zinc sensitivity is only weakly affected by the A (or C) mutation (~ 2 -fold shift in IC_{50} ; Fig. 5), excluding the possibility that Tyr231 mutations exert their effects through an indirect global structure disruption.

We also assessed the proton sensitivity of receptors containing mutations at Val262 and Tyr231. Indeed, protons are potent inhibitors of NR1/NR2B receptors (H^+ IC_{50} of pH ~ 7.3 close to the physiological pH), and ifenprodil has been proposed to inhibit receptor activity through an enhancement of tonic proton inhibition (Mott et al., 1998). It could therefore be that the reduced ifenprodil inhibition that we observed on the mutant receptors reflects a decrease in pH sensitivity. To verify whether this is the case, we determined the pH sensitivity of the mutant receptors that yield the strongest decrease in ifenprodil sensitivity [i.e., NR1/NR2B-V262F and NR1/NR2B-Y231A (≥ 50 -fold shift in ifenprodil IC_{50} ; Table 1)]. We also determined pH sensitivity of receptors containing either NR2B-D101A or NR2B-F176A, two mutations that we previously showed to strongly affect ifenprodil sensitivity (Perin-Dureau et al., 2002). As shown in Figure S1, none of these mutations have a significant effect on pH sensitivity [pH IC_{50} of 7.52 ($n = 4$), 7.46 ($n = 4$), 7.50 ($n = 4$), and 7.39 ($n = 3$) for NR1/NR2B-D101A, NR1/NR2B-F176A, NR1/NR2B-Y231A, and NR1/NR2B-V262F receptors, respectively, versus 7.45 ($n = 4$) for wt NR1/NR2B receptors]. These data demonstrate that the mutations do not alter ifenprodil sensitivity secondary to changes in pH sensitivity. Rather, they provide further validation that the identified residues are likely to be true binding residues and do not act through indirect gating effects.

Because in the two proposed models, both Val262 and Tyr231 make hydrophobic interactions with the aromatic rings of ifenprodil (ring A in orientation 1 or ring B in orien-

tation 2), the above results are of little help to discriminate between orientations 1 and 2. The situation is strikingly different concerning the interactions of the hydroxyl group of ring B. In orientation 2, the ifenprodil phenol moiety is proposed to make hydrogen bonds with two residues from the hinge, Gln153 and Tyr282. The mutation of Gln153 into an alanine or a cysteine, to prevent formation of these hydrogen bonds, results in only a very mild shift in ifenprodil sensitivity (1.5- to 2-fold shift in IC_{50} ; Table 1 and Figure S2A). Likewise, the mutation of Tyr282 into a cysteine, a serine, or a tryptophan induces a modest, but stronger, decrease in ifenprodil sensitivity (~ 4 -fold shift in IC_{50} ; Figure S2C). As the presence of a H-bond donor at the para position of ring B is critical for ifenprodil-like compounds activity (Chenard

and Menniti, 1999), and as in orientation 2, ifenprodil phenolic group is in a rather hydrophobic environment, the mutations of either Gln153 or Tyr282 would, in orientation 2, be expected to induce relatively strong effects on ifenprodil sensitivity. This is clearly not what we observed, indicating that the model of ifenprodil binding in orientation 2 is likely to be incorrect. It is noteworthy that the mutations of residues Gln153 or Tyr282 induce a decrease in the maximal level of inhibition produced by ifenprodil (Supplemental Fig. S2, A and C). The larger effect is obtained with the Y282S mutation, which yields a maximal inhibition of 66% (versus 95% on wt NR1/NR2B receptors; Supplemental Fig. S2C). However, because zinc inhibition is not affected by these hinge mutations (Supplemental Fig. S2, B and D), it is likely that

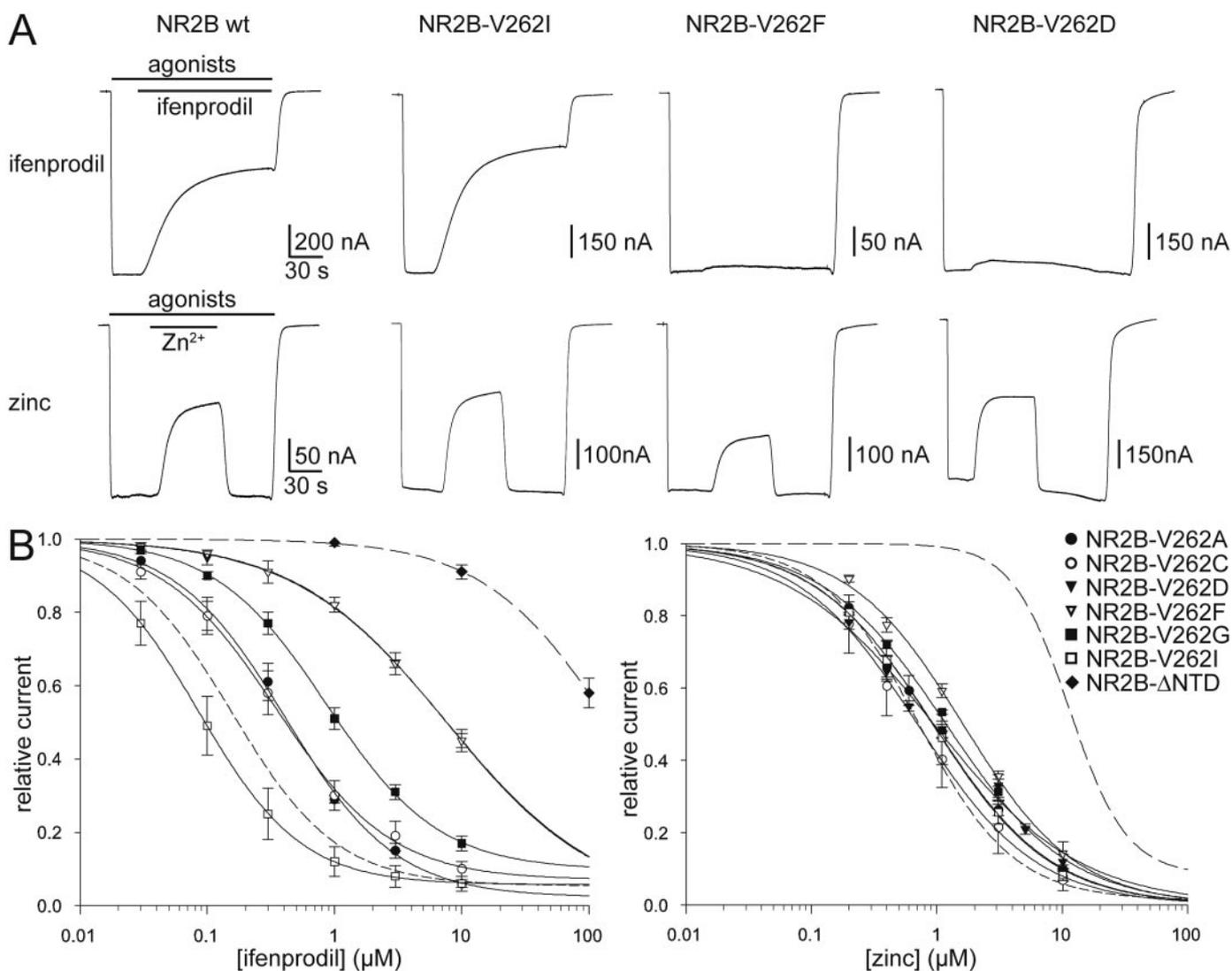


Fig. 4. NR2B-Val262, a new key residue selectively controlling ifenprodil inhibition. **A**, typical current traces obtained from oocytes coexpressing the wt NR1 subunit with wt or mutated Val262I, V262F, or V262D NR2B subunits. Ifenprodil was applied at a concentration of 300 nM and zinc at 1 μ M, each during an application of agonists. The bars above the current traces indicate the duration of agonists, ifenprodil, and zinc applications. Note that the inhibition produced by 1 μ M zinc is only weakly affected by any of the three mutations [inhibition of $54 \pm 8\%$ ($n = 3$), $41 \pm 2\%$ ($n = 4$), and $51 \pm 2\%$ ($n = 3$) for NR2B-Val262I, V262F, and V262D mutations, respectively, versus $61 \pm 4\%$ for wt receptors ($n = 12$)]. In contrast, ifenprodil sensitivity is strongly reduced by the NR2B-V262F or D mutations, whereas it is slightly increased with the NR2B-Val262I mutation. **B**, ifenprodil (left) and zinc (right) concentration-response curves of NR1/NR2B receptors containing different substitutions at the NR2B-Val262 position. The dashed curves are the fits of the ifenprodil or zinc dose-response curves of wt NR1/NR2B receptors (short dashes) and receptors truncated for their entire NR2B-NTD (NR1/NR2B- Δ NTD, long dashes; see Rachline et al., 2005). Experimental data points obtained with NR1/NR2B- Δ NTD receptors are displayed as closed diamonds. Each data point is the mean value of at least three different cells. The estimated values of IC_{50} are listed in Table 1. Note that NR2B-Val262 mutations selectively and differentially affect ifenprodil sensitivity. Note also that the experimental data points and the fits of the NR1/NR2B-V262D (\blacktriangle) and NR1/NR2B-V262F (\blacktriangle) mutant receptor dose-response curves overlap.

these do not modify the intrinsic gating properties of the receptor but rather the binding of ifenprodil per se. The mechanism underlying the partial nature of NR1/NR2B receptor inhibition by ifenprodil-like compounds is still un-

known, but it is conceivable that, in these modified NR2B NTDs, ifenprodil adopts a slightly different position resulting in a decreased level of inhibition of the receptors.

To test the validity of the ifenprodil binding model with

TABLE 1
Effects on ifenprodil and zinc sensitivity of various mutations in NR2B NTD

NR2B Mutants	Ifenprodil			Zinc		
	IC ₅₀ μM	Mutant/wt Ratio	n	IC ₅₀ μM	Mutant/wt Ratio	n
wt	0.16 ± 0.01		9	0.70 ± 0.08		15
T76						
T76A	2.3 ± 0.8	15	3	0.60 ± 0.06	0.8	6
T76C	2.5 ± 0.1	16	3	0.9 ± 0.1	1.3	3
T76S	0.43 ± 0.01	2.8	3	0.40 ± 0.05	0.6	3
D77						
D77C	2.8 ± 0.2	18	4	0.9 ± 0.3	1.3	5
Q153						
Q153A	0.22 ± 0.04	1.4	7	0.8 ± 0.3	1.1	4
Q153C	0.28 ± 0.02	1.8	7	0.8 ± 0.3	1.1	6
D206						
D206A	4 ± 1	30	7	2.6 ± 0.4	3.7	6
D206C	5 ± 2	30	4	1.4 ± 0.1	2.0	5
D206E	1.5 ± 0.1	10	8	0.74 ± 0.02	1.1	6
D206F	7 ± 4	45	3	2.0 ± 0.1	2.9	3
D206K	13 ± 15	80	3	4.7 ± 0.2	6.7	3
Y231						
Y231A	53 ± 10	350	3	1.6 ± 0.4	2.3	3
Y231C	26 ± 8	170	6	1.6 ± 0.6	2.3	6
V262						
V262A	0.41 ± 0.04	2.6	3	0.9 ± 0.1	1.3	3
V262C	0.35 ± 0.03	2	3	0.7 ± 0.1	1.0	4
V262D	8 ± 2	50	4	0.9 ± 0.1	1.3	3
V262F	8 ± 2	50	3	1.6 ± 0.3	2.3	4
V262G	0.87 ± 0.04	5.6	5	1.2 ± 0.3	1.7	3
V262I	0.09 ± 0.001	0.55	5	0.9 ± 0.1	1.3	3
Y282						
Y282C	0.8 ± 0.2	5	9	0.7 ± 0.1	1	7
Y282S	0.7 ± 0.1	4.5	3	1.6 ± 0.6	2.3	3
Y282W	0.24 ± 0.02	1.5	4	0.9 ± 0.1	1.3	3
NTD deletion NR2B-ΔNTD	145 ± 3	900	3	11 ± 1	16	3

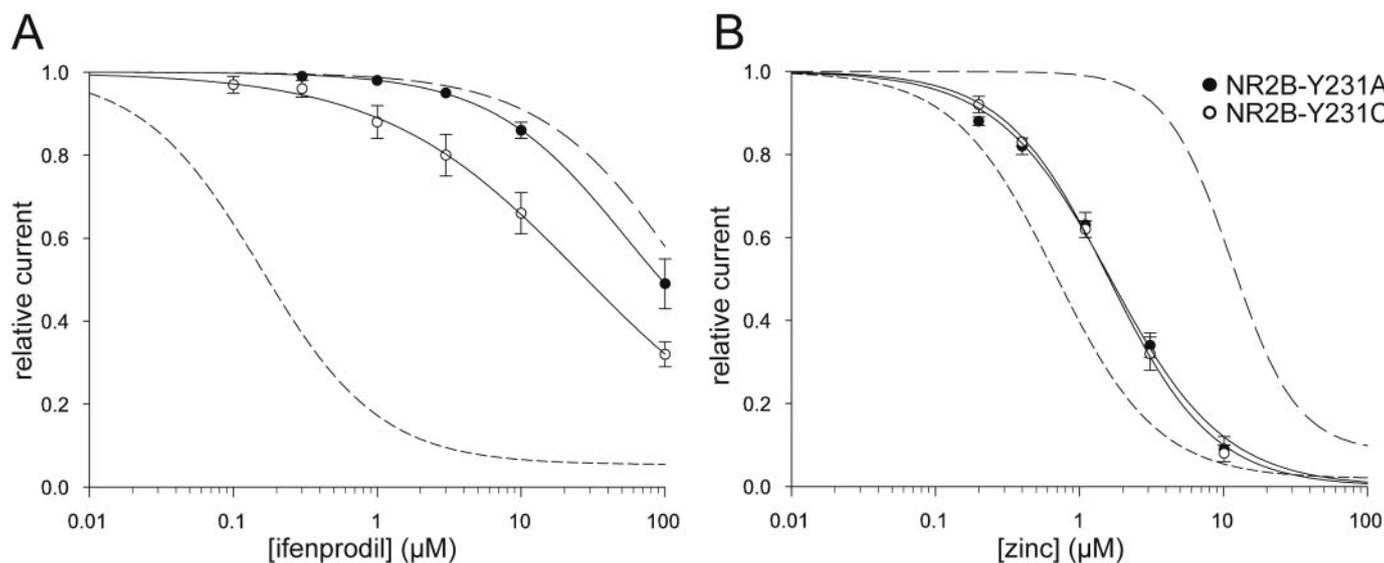


Fig. 5. NR2B-Tyr231, an additional key residue controlling ifenprodil inhibition. Ifenprodil and zinc sensitivity of NR1/NR2B receptors substituted with different residues at the NR2B-Tyr231 position. In each panel, the dashed curves are the fits of the ifenprodil or zinc dose-response curves of wt NR1/NR2B receptors (short dashes) and NR1/NR2B-ΔNTD receptors (long dashes). Each data point is the mean value of at least three different oocytes. A, ifenprodil concentration-response curves B, zinc concentration-response curves. The estimated values of IC₅₀ are listed in Table 1. Note that the shift in ifenprodil sensitivity produced by the NR2B-Y231A mutation is almost as large as the shift produced by the deletion of the entire NR2B NTD.

orientation 1, the three residues that make hydrogen bonds with the phenol moiety in this orientation, Thr76, Asp77 and Asp206, were mutated. To verify that Asp206 actually acts as a H-bond acceptor, its polarity was either conserved (glutamate mutation) or changed by introducing hydrophobic (alanine and phenylalanine), neutral polar (cysteine) or positively charged (lysine) residues. Figure 6, A and B, shows that such mutations of Asp206 affect both ifenprodil and zinc sensitivity but, depending on the substitution, the amplitude of the effects differs significantly between the two ligands. For zinc sensitivity, we observed a strong correlation with the side chain charge. First, mutating the aspartate into a glutamate (charge conservation) has no effect on zinc sensitivity [IC_{50} of $0.67 \mu\text{M}$ for NR2B-D206E ($n = 3$) versus $0.70 \mu\text{M}$ for wt receptors ($n = 15$); Fig. 6B]. Second, the mutant receptors become progressively less sensitive to zinc as the

charge of the substituting amino acid becomes more positive (Glu \rightarrow Cys, Ala, Phe \rightarrow Lys; Fig. 6B and Table 1). It is noteworthy that the mutations of the homologous NR2A residue Asp207 had the same phenotype on high-affinity zinc sensitivity of NR1/NR2A receptors (Paoletti et al., 2000). NR2A-Asp207, located at the entrance of the NTD cleft, was then proposed to provide a favorable electrostatic environment for zinc to access the cleft. Therefore, we propose that, concerning zinc sensitivity, Asp206 in NR2B may have a similar attractive role. In contrast to the effects seen on zinc sensitivity, we observed that ifenprodil sensitivity is greatly reduced by mutations at Asp206, whatever the nature of the mutation (Fig. 6A). In particular, substitution of Asp206 by a glutamate, a residue that is still capable of forming H-bonds but is one carbon longer, induces a 10-fold decrease in ifenprodil sensitivity (Table 1). Mutation of Asp206 into a weakly

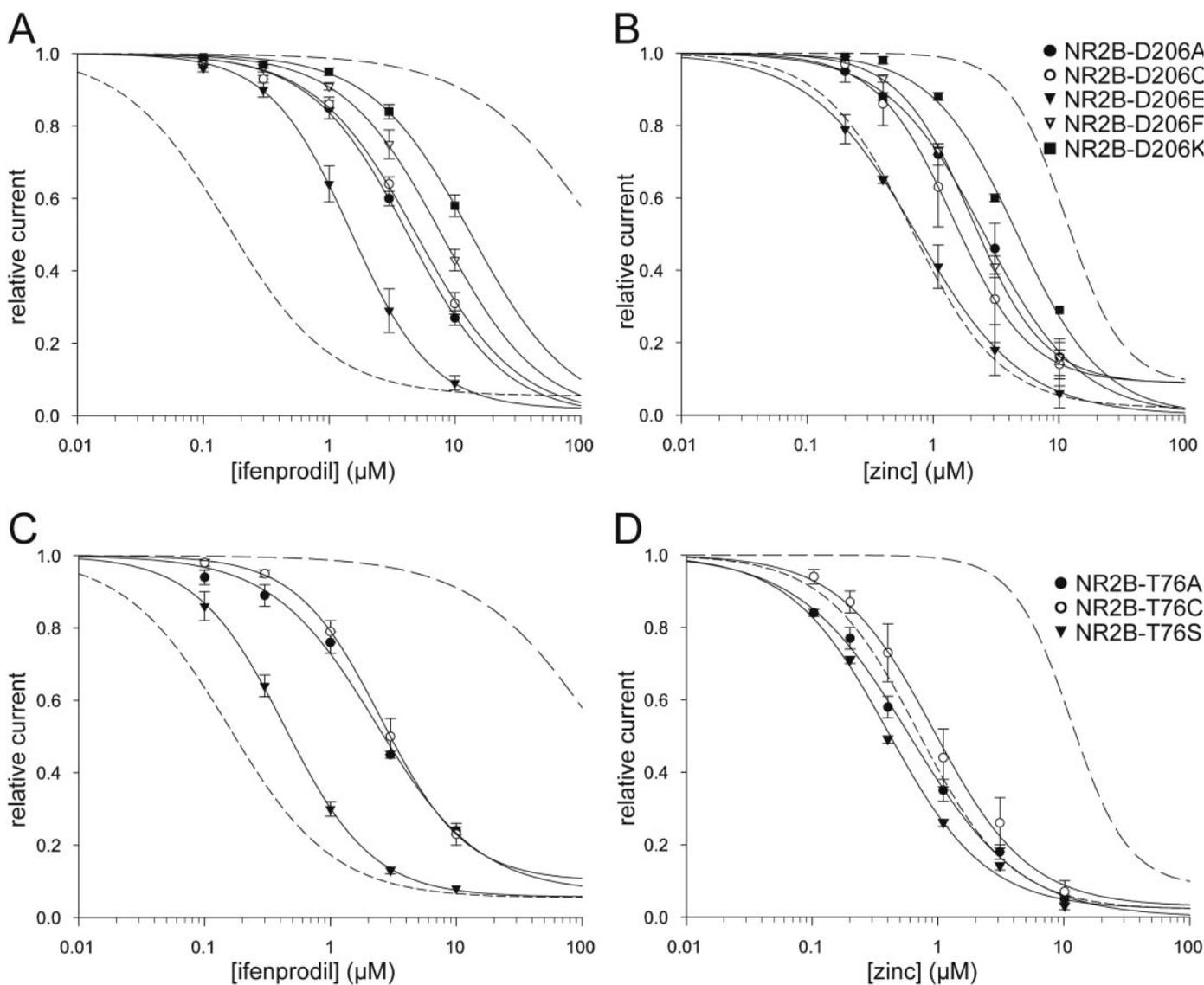


Fig. 6. Effects of NR2B-Asp206 and NR2B-Thr76 mutations on ifenprodil and zinc sensitivity. Ifenprodil and zinc sensitivity of NR1/NR2B receptors substituted with different residues at NR2B-Asp206 and NR2B-Thr76 positions. In each panel, the dashed curves are the fits of the ifenprodil or zinc dose-response curves of wt NR1/NR2B receptors (short dashes) and NR1/NR2B- Δ NTD receptors (long dashes). Each data point is the mean value of at least three different oocytes. A, ifenprodil concentration-response curves of NR1/NR2B receptors mutated at the NR2B-Asp206 position. B, zinc concentration-response curves of NR1/NR2B receptors mutated at the NR2B-Asp206 position. C, ifenprodil concentration-response curves of NR1/NR2B receptors mutated at the NR2B-Thr76 position. D, zinc concentration-response curves of NR1/NR2B receptors mutated at the NR2B-Thr76 position. The estimated values of IC_{50} are listed in Table 1.

polar (cysteine) or hydrophobic residue (alanine, phenylalanine) produces a much larger decrease in ifenprodil sensitivity (30–45-fold shift in IC_{50} ; Table 1 and Fig. 6A). The largest effect is obtained by the mutation of Asp206 into a lysine (80-fold shift in IC_{50}), a bulky and positively charged residue. These results strongly suggest that, in addition to its attractive electrostatic role, Asp206 is also likely to directly contact the ifenprodil molecule through polar bonds. This result clearly favors orientation 1 model, in which Asp206 makes a charge-dipole interaction with ifenprodil B-ring hydroxyl group.

The mutation of Asp77 into a cysteine selectively reduces ifenprodil sensitivity (IC_{50} 18-fold higher than for wt receptors), with no change in zinc sensitivity (Supplemental Fig. S3 and Table 1). Besides, the mutation of Thr76 into an alanine or a cysteine has a similar phenotype (15-fold shift in ifenprodil IC_{50} ; very little change in zinc IC_{50} ; Fig. 6, C and D, and Table 1). These results suggest that Thr76 and Asp77 are likely to interact with ifenprodil. Moreover, mutating Thr76 into a serine, a residue with a conserved alcohol function, but without the methyl group of threonine, only slightly affects ifenprodil sensitivity [<3 -fold shift in ifenprodil IC_{50} ; Fig. 6C and Table 1], indicating that the alcohol function of the threonine is an important determinant of ifenprodil sensitivity. Thus, Thr76, through its hydroxyl moiety, is likely to make a hydrogen bond with ifenprodil. Altogether these results show that Asp206, Thr76, and Asp77 control ifenprodil inhibition, whereas Gln153 and Tyr282 do not. These results are fully consistent with ifenprodil binding in the NTD of NR2B in orientation 1.

Orienting Ifenprodil in Its Binding-Pocket Using the Cysteine Affinity Labeling Approach. If site-directed mutagenesis experiments can provide reliable information regarding which residues control sensitivity to a ligand, they are less powerful to discriminate between residues actually directly binding the ligand from residues having more distant structural effects. In our case, the presence of a second ligand of very different chemical nature, the zinc ion, which also binds into NR2B NTD (Rachline et al., 2005), enables us to distinguish residues selectively controlling ifenprodil inhibition (considered truly “binding” residues) from residues controlling both ifenprodil and zinc inhibition (considered potential “structural” residues). However, for ifenprodil-selective residues, an uncertainty remains whether they are directly in contact with ifenprodil or they belong to its second coordination sphere. Furthermore, site-directed mutagenesis experiments give no direct information about the precise part of the ligand interacting with the highlighted residue. To probe for direct interactions between a precise region of the NTD and a specific part of the ifenprodil molecule, and thus help orientate unequivocally this compound into its binding pocket, we used the cysteine affinity labeling approach (Foucaud et al., 2001). This technique involves the formation of a covalent bond between a cysteine-reactive ligand derivative and a cysteine-substituted receptor, provided that the ligand reactive group and the cysteine are in close proximity (Fig. 7A) (Foucaud et al., 2001). This strategy was previously applied to explore the glycine-binding site of the NMDAR NR1 subunit (Foucaud et al., 2003), and the results were remarkably consistent with the crystal structure of the NR1 agonist-binding domain (Furukawa and Gouaux, 2003). In our case, we used the reactive ifenprodil

derivatives previously developed by Alarcon et al. (2008) and particularly *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide, in which the phenolic hydroxyl group was replaced by a cysteine-reactive chloroacetamide group (Fig. 7). *N*-{4-[2-(4-Benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide displays the required reactivity toward cysteine, good stability in solution and significant NR2B NTD-mediated antagonist properties at wt NR1/NR2B receptors (IC_{50} of 14 μ M; Alarcon et al., 2008). Furthermore, the two point mutations, NR2B-D101A and V262D, that strongly decrease ifenprodil sensitivity (see Table 1), also markedly reduced sensitivity of NR1/NR2B receptors for *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide [inhibition by 10, 30 and 100 μ M of 8 ± 2 , 15 ± 7 , and $20 \pm 3\%$, respectively, for NR1wt/NR2B-D101A receptors ($n = 3$), of 12 ± 8 , 17 ± 5 , and $28 \pm 9\%$, respectively, for NR1wt/NR2B-V262D receptors ($n = 3$) versus 41 ± 14 , 56 ± 11 , and $81 \pm 6\%$, respectively, for wt NR1/NR2B receptors ($n = 5$)], indicating that *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide is likely to share the same anchoring points as ifenprodil in the NR2B NTD cleft.

To test which of the orientations (orientation 1 or 2) is used by *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide, we substituted residues Thr76, Leu205, Asp206, Gln153 and Val262 of the NR2B NTD by cysteines. We selected these residues because there are predicted to contact (or be in close vicinity to (Leu205)) the reactive antagonist in the different docking orientations (Fig. 2). The initial NMDA current (I_0) was measured on oocytes expressing NMDARs containing one of the above cysteine mutations. Oocytes were then incubated in a solution containing a high concentration of *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide (500 μ M, close to saturation), and after 30 min of incubation, they were washed to remove any reversible binding of *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide. After washing, the NMDA current was measured again (I_{incub}). We expected the ratio I_{incub}/I_0 to be less than 1 if *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide induced an irreversible labeling of the cysteine-modified receptors and to be close to 1 if no irreversible labeling occurred. We were surprised to find, however, that in preliminary control experiments, long incubation times with *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide, even with the nonreactive ifenprodil molecule, could produce a long-lasting inhibition of wt NR1/NR2B receptors, an effect that is mediated by the binding of the ligand to the NR2B NTD (see *Materials and Methods*). To circumvent this undesired “side” effect, we systematically compared the effects of the reactive ligand on cysteine-substituted NMDARs with those on NMDARs substituted with an alanine at the same position. As an additional control, we checked that, at the positions tested, receptors mutated into an alanine or a cysteine had similar ifenprodil IC_{50} values. This was indeed the case (Table 1). With these precautions in hand, a significant difference between the I_{incub}/I_0 ratios of alanine- and cysteine-substituted receptors is expected to account for an irreversible labeling of the targeted position. As shown in Fig. 7B, at positions lining the entrance of the NR2B NTD cleft (i.e., Leu205 and Asp206 from lobe II and Thr76 from lobe I), a significant difference is observed between the I_{incub}/I_0 ratio of the alanine and the

cysteine mutant. This result suggests that *N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide could specifically react with the NTD at these positions. On the contrary, no significant difference is observed at positions Gln153 and Val262, close to the hinge. These results are strongly in favor of the orientation 1 docking model in which ifenprodil phenol group is near the entrance of the binding cleft, whereas its phenyl group points in the opposite direction toward the hinge. These results also provide further support toward a direct interaction of the residues Thr76 and Asp206 with the phenolic hydroxyl group of ifenprodil, as proposed in the orientation 1 model.

Discussion

In the present work, we delineate the structural determinants that are responsible for the high-affinity binding of ifenprodil on the NR2B subunit. For that purpose, we have built 3D homology models of ifenprodil docked in its binding pocket and have subjected these models to an extensive experimental validation process based on site-directed mutagenesis and cysteine affinity labeling. A number of important features emerge from this study: first, as evidenced by the stable docking, ifenprodil fits well into the central crevice of the NR2B N-terminal domain modeled according to a

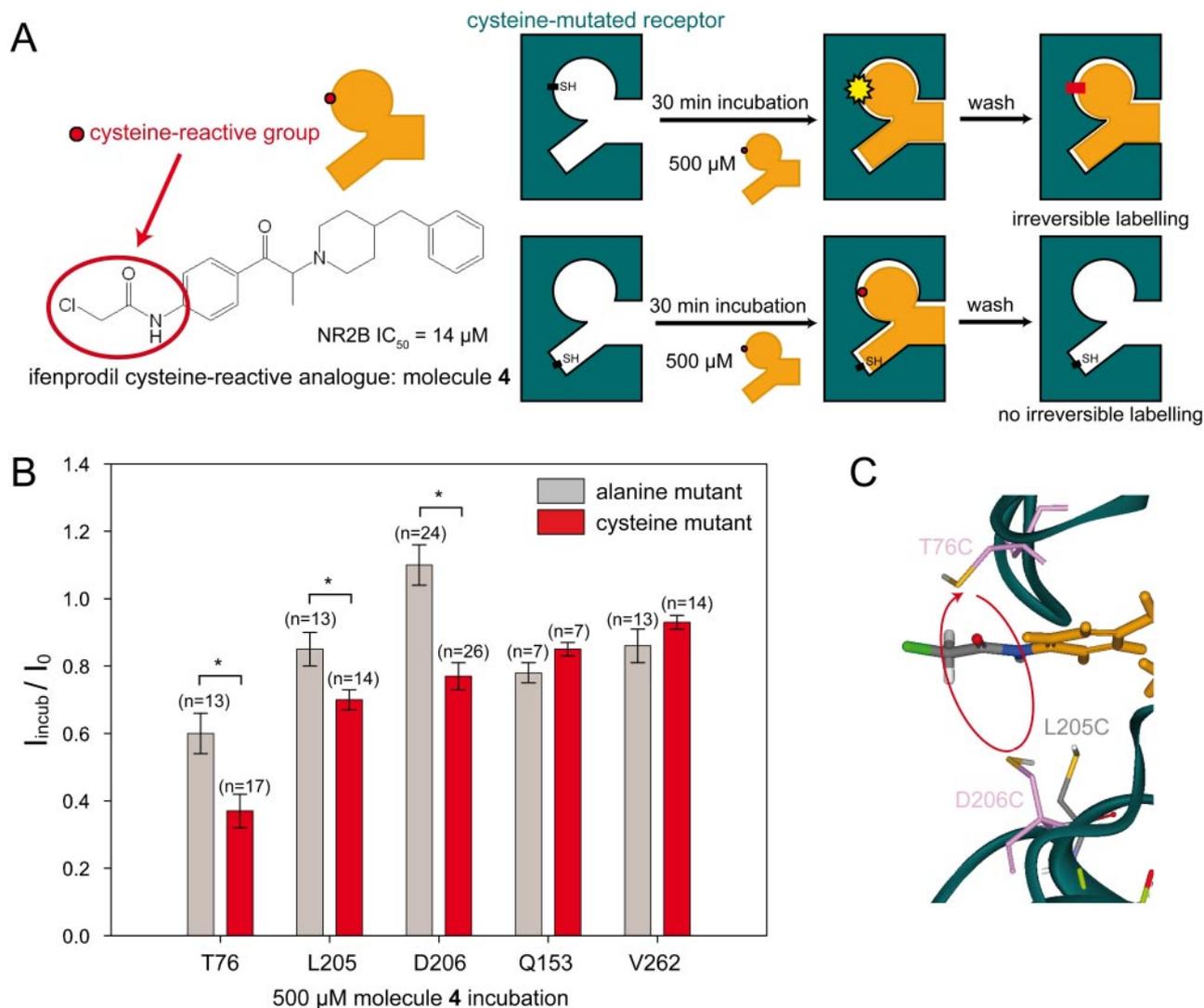


Fig. 7. Orienting ifenprodil in its binding pocket using cysteine affinity labeling. **A**, principle of the cysteine affinity labeling approach. This technique is based on the formation of a covalent bond between a cysteine-reactive ligand (schematized in orange with a red circle representing the cysteine-reactive group) and a cysteine-modified receptor (schematized in green). If the cysteine and the cysteine-reactive group are in close proximity, the formation of a covalent bond leads to an irreversible labeling of the receptor (in our case an irreversible inhibition). In the present work, we used *N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide, a cysteine-reactive ifenprodil analog containing a cysteine-reactive chloroacetamide group on the *para*-position of the B-ring (circled in red). **B**, affinity labeling results. For each position, the I_{incub}/I_0 ratio of the cysteine mutant is represented as a red bar and that of the corresponding alanine mutant as a gray bar. Error bars represent the standard error of the mean. The numbers of oocytes used for each construction are shown in parentheses above the bars. We used a Student *t* test to probe for a significant difference between the I_{incub}/I_0 ratios of the alanine and the cysteine substitution at the same position (*, $P < 0.05$). **C**, representation of *N*-[4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl]-2-chloro-acetamide (orange sticks) bound to the NR2B NTD in orientation 1, with cysteine residues at positions NR2B-Thr76, Asp206 (pink carbon chains) and Leu205 (gray carbon chain) introduced by *in silico* mutagenesis. Sulfur atoms are displayed in yellow and hydrogen atoms in white. Hydrogen atoms of the cysteine carbon chains are not represented. The chloroacetamide group (displayed as sticks colored as follows: carbon in gray, chloride in green, hydrogen in white, nitrogen in dark blue, and oxygen in red) displays rotational mobility (red curved arrow) and thus can potentially make covalent bonds with either lobe I (T76C) or lobe II (D206C and L205C) residues.

closed conformation of the structurally related bilobate agonist-binding domain of mGluR1. Second, despite being a rather symmetrical molecule, ifenprodil likely adopts an unique and well-defined orientation within this crevice. Third, ifenprodil seems to interact with residues of both NTD lobes, strongly suggesting that it stabilizes a closed-cleft conformation of NR2B NTD, much like activating ligands at other LIVBP-like Venus-flytrap domains (Kunishima et al., 2000; Magnusson et al., 2004; Acher and Bertrand, 2005). Fourth, high-affinity ifenprodil binding is achieved through multiple ligand-protein interactions, involving electrostatic and hydrogen bonds together with Van der Waals contacts distributed all along the ifenprodil molecule.

Based on our models, we have identified, in site-directed mutagenesis experiments, five new NR2B NTD residues that are key for high-affinity ifenprodil inhibition of NR1/NR2B receptors: Thr76, Asp77, Asp206, Tyr231, and Val262. Moreover, by performing multiple side-chain substitutions at these positions and by systematically controlling for specificity toward ifenprodil versus zinc, the other known NR2B NTD ligand (Rachline et al., 2005), we obtained strong support for direct interaction between these residues and ifenprodil. This conclusion was strengthened further in the case of NR2B residues Asp101, Phe176, Tyr231, and Val262 by showing that mutations at these positions that strongly affect ifenprodil sensitivity (up to >300-fold shift in ifenprodil IC₅₀, the case of the NR2B-Y231A mutation) do not alter pH sensitivity. All together, these mutagenesis results provide a clear experimental validation of our proposed models. They also allowed us to propose an orientation of the ifenprodil molecule in its binding pocket, something that the modeling alone could not achieve. Ifenprodil binds in an extended conformation, almost perpendicular to the plane of the NTD hinge with its phenyl group (ring A) located close to the NTD hinge and its phenol moiety (ring B) pointing toward the entrance of the cleft. We obtained an additional confirmation that this orientation is likely to be functionally relevant by performing cysteine affinity labeling experiments. Indeed, experiments using *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide, a thio-reactive ifenprodil analog functionalized at the level of the phenol hydroxyl group, revealed that this compound can react with cysteines introduced at the entrance of the NTD cleft but not with cysteines deep in the cleft near the hinge. Moreover, the fact that *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide labels residues from both lobes of the NTD (Thr76 from lobe I and Leu205 and Asp206 from lobe II) strongly supports a model in which binding of ifenprodil promotes closure of NR2B NTD.

However, if mutagenesis experiments gave effects on ifenprodil inhibition easily interpretable, cysteine affinity labeling experiments were harder to settle. First, although applied at a concentration close to saturation, *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide induced only a partial irreversible labeling of the cysteine-mutated receptors as judged by the changes in current amplitude (specific inhibition from 18% for the L205C mutation to 38% for the T76C mutation; Fig. 7). The partial nature of the irreversible labeling may be due to the low reactivity of *N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide with free cysteines in solution ($t_{1/2} = 114$ min in an excess of *N*-acetylcysteine methyl-ester; Alarcon et al.,

2008), although (*N*-{4-[2-(4-benzyl-piperidin-1-yl)-propionyl]-phenyl}-2-chloro-acetamide is expected to react much faster once bound in NR2B NTD because of presumably close proximity of the thiol reactive group with the introduced cysteines. We also attempted affinity labeling experiments with another cysteine-reactive ifenprodil analog: compound **12** from Alarcon et al. (2008), containing an isothiocyanate group at the para position in ring A. If ifenprodil does actually bind in NR2B NTD with orientation 1, compound **12** was expected to specifically label cysteines introduced at positions close to the hinge. We were disappointed, however, that we could not find any position in NR2B NTD at which irreversible labeling with this molecule could be observed, either at positions close to the hinge or at positions close to the entrance of the cleft. Assuming our model is valid, this lack of effect could be due to strong structural constraints: from one side, the low intrinsic rotational mobility of the isothiocyanate group, and from the other, the narrowness of the binding pocket near the NTD hinge. These two effects combined may sharply decrease the probability to find a position at which a cysteine could be properly orientated to react with molecule **12** isothiocyanate group.

A model of ifenprodil binding in NR2B NTD, with no experimental validation, was already proposed by Marinelli et al (2007). Our model shares only few similarities with theirs, in which ifenprodil has an almost perpendicular orientation. This difference of docking orientation could be explained by the use of a different sequence alignment, especially for some loops located in the binding cleft, which could give rise to a different shape of ifenprodil binding-site. Marinelli et al. (2007) found, as we did, an interaction of Asp101 with both the central positive amino group and the linker's hydroxyl group. They also highlight Val262 as a residue near the ifenprodil molecule. However, in their case, Val262 interacts with the phenol moiety (ring B), whereas in ours it interacts with the other aromatic ring, the phenyl moiety (ring A). There are multiple points in the model of Marinelli et al. (2007) that are difficult to reconcile with experimental results obtained in this and previous studies. For instance, their model does not explain the critical roles of Ile150 and the newly found Tyr231 residue, two residues that produce the largest observed shifts in ifenprodil sensitivity when mutated into alanine with no or little effect on zinc sensitivity (Perin-Dureau et al., 2002 and this study). Furthermore, ifenprodil phenol hydroxyl group is proposed to be in close proximity with Asp265. An alanine mutation of this residue, located in a rather apolar environment, should therefore have a substantial effect on ifenprodil sensitivity. However, such mutation has no effect on ifenprodil sensitivity (Perin-Dureau et al., 2002). These data obviously do not substantiate the model obtained by Marinelli et al. (2007).

An interesting and striking observation is that almost all the residues that in our model line the ifenprodil binding pocket by directly interacting with the ligand are conserved in NR2A NTD (but not in NR2C or NR2D NTD). Of 13 contacting residues, 11 are identical between the two subunits, one is homologous (Thr233, which is a serine in NR2A), and one is absent (Val42, which is a glycine in NR2A) (Fig. 1). The question arises then of why ifenprodil does not affect activity of NR1/NR2A receptors by binding to NR2A NTD as the zinc ion does. A single residue could make the difference. For instance, it is conceivable that ifenprodil cannot enter the NR2A NTD crevice because of steric hindrance produced by a

bulky NR2A-specific residue protruding in the crevice. A potential candidate residue is NR2A-H42, which is replaced by a serine in NR2B. However, substituting this histidine into a shorter alanine does not confer ifenprodil sensitivity (L. Mony and P. Paoletti, unpublished observations). The most divergent region between NR2A and NR2B NTD is the $\beta 1$ - $\alpha 1$ region, which includes NR2B-V42. Again, replacing this entire region of NR2A-NTD with that of NR2B fails to confer ifenprodil sensitivity to the modified NR1/NR2A receptor (L. Mony and P. Paoletti, unpublished observations). Functional studies using NTD chimeric NR2 subunits and binding studies on the isolated NR2B NTD (Perin-Dureau et al., 2002; Wong et al., 2005) indicate that the molecular determinants underlying high-affinity ifenprodil binding are fully embedded in the NR2B NTD with no contribution from NR2B-specific residues outside this domain. It is possible therefore that ifenprodil selectivity for NR2B NTD originates from a limited number of NR2B-specific residues scattered throughout the NTD sequence and that are key for correct positioning of residues directly interacting with the ifenprodil molecule.

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