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EFFECTS OF IFENPRODIL ON THE GATING OF 
NR1/2B NMDA RECEPTORS

A Master’s thesis by

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ABSTRACT

The extracellular amino terminal domain (ATD) of the NR2 subunits differentially controls NMDA receptor activity. Ifenprodil binds to the ATD of NR1/NR2B receptors inhibiting the receptors. To delineate the effects of ifenprodil on NR1/NR2B receptor gating, we recorded steady state currents from cell attached and excised outside out patches at pH7.9. NR1/NR2B receptors were found to exhibit modal gating in both forms of recording which was unaffected by ifenprodil. Ifenprodil increased the occupancy of the receptor in long-lived shut conformations hence reducing the open probability of receptor with no change in the mean open time. We found a negative correlation between the open and shut intervals in NR1/NR2B recordings from cell attached patches containing one active channel similar to those previously reported for NR1/NR2A receptors. In order to further understand actions of ifenprodil we fitted the single channel data to previously proposed models for NMDA receptor gating. Log likelihood criteria suggested that a cyclic model containing two uncoupled open states (Schorge et al. 2005) fitted the data better compared to a linear model with sequential open states. Ifenprodil modified multiple gating steps when fitted to an ‘uncoupled cyclic’ model and promoted receptor desensitization. In contrast a single gating step was modified when single channel data was fitted to the ‘linear’ gating model. Our results suggest an allosteric cross talk between the NR1 and NR2 subunits of the NMDA receptors.
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DEDICATION:

This dissertation, the manuscript and my career in Neuroscience is dedicated to the Indian culture and all my teachers and professors. Throughout my life, they have inculcated in me an insatiable quest for knowledge.
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Chapter 1

Introduction
A. **Glutamate receptors:**

The development and working of a human brain rests on the organization and functioning of neurons. These neurons have the ability to form new connections, ‘synapses’ with other neurons. The strength, organization and functioning of these synapses constantly changes via our new experiences. It is this synaptic plasticity that empowers us to respond uniquely and appropriately to the varied environments.

At the synapses, neurotransmitters released from presynaptic nerve terminals activate postsynaptic receptors. Hence these neurotransmitters convey excitatory or inhibitory messages from one neuron to another. Glutamate is a neurotransmitter that mediates the vast majority of excitatory neurotransmission in the central nervous system. Glutamate receptors are ubiquitously present in the central nervous system.

There are of two classes of glutamate receptors: ionotropic and metabotropic. The ionotropic glutamate receptors (iGluRs) are ligand gated ion channels. They are implicated in almost all aspects of nervous system functions. Calcium entry through the iGluRs plays an important role in synaptic plasticity which is the basis of learning and memory (Dingledine et al., 1999). Based on sequence homology and pharmacology, there are four classes of glutamate receptors -

1. *N*-methyl *D*-aspartate Receptors: NMDAR,
2. *α*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors: AMPAR,
3. Kainate receptors and
4. Delta receptors
**B. NMDA receptors:**

N-methyl-D-aspartate (NMDA) receptors are a member of the glutamate family of ionotropic receptors. They are highly expressed in the central nervous system. NMDA receptors (NMDAR) mediate the slow component of the synaptic transmission. Like other members of the glutamate receptor family, they are involved in fundamental processes like memory formation and learning. Dysregulation of NMDA receptor function leads to pathophysiological conditions like stroke, mental illness and neurodegenerative disorders (Dingledine et al., 1999).

NMDA receptors are unique amongst the iGluRs as they are ligand gated as well as voltage dependent. They have a dual agonist requirement in that glutamate and glycine both are required to activate the channels (Kleckner and Dingledine 1988). NMDA receptors are voltage dependence as the channel pore is blocked by magnesium. During synaptic transmissions, the
rapid calcium influx via the AMPAR depolarizes the neurons, relieving the voltage dependent magnesium block.

C. Crystal structure of NMDA receptors:

The architecture of the NMDA receptors, on the basis of GluR2 receptor X-ray structure, was suggested recently (Sobolevsky et al. 2009). Unlike APMA and Kainate receptors, NMDA receptors are obligate heterotetramers (Behe et al., 1995). AMPA and Kainate receptors form homotetramers however native receptors are mostly heterotetramers. NMDA receptors assemble as tetramers and are composed of two obligatory glycine binding NR1 subunits and two glutamate binding NR2 subunits. They are arranged as dimers of dimers, each dimer composed of one NR1 and one NR2 subunit (Figure 2). The crystal structure predicts that NMDA receptors have a 1-2-1-2 subunit arrangement. (Figure 3)
Figure 2: A side view of a NR1/NR2B NMDAR dimer. The Amino terminal domain and Ligand binding domain are located in the extracellular side, the Carboxy terminal domain facing the intracellular side and the four transmembrane domains are embedded in the cell membrane.

Figure 2 depicts the four domains that each NMDAR subunit is composed of. They are as follows:

(i) Amino Terminal Domain (ATD)

(ii) Ligand Binding Domain (LBD)

(iii) Transmembrane Domain (TM 1-4)

(iv) Carboxy Terminal Domain (CTD)
Figure 3: Crystal structure of iGluRs: a view of the ‘broad’ face of the receptor, perpendicular to the two-fold axis of overall symmetry. Each subunit is depicted in a different color. In context of NMDAR: A, C represent the NR1 subunits whereas, B and D represent the NR2 subunits. Domain layers viewed from top of the receptor, parallel to the overall two-fold axis, illustrate the dimer-dimer contacts. The NR2 subunits are in contact with each other at the amino terminal domain (ATD) layer. The NR1 subunits are in contact at the ligand binding domain (LBD) layer. The receptor exhibits an overall axis of 2-fold symmetry which systematically transitions into a 4-fold symmetry within the transmembrane domain (TMD) (Figure is modified from Sobolevsky et al. 2009)

One of the major hurdles in the past to study the structure-function of NMDA receptors has been the uncertainty regarding the NMDA receptor subunit arrangement. The recently proposed crystal structure provides a basis for the development of various conceptual models of NMDAR gating and also better structure function understanding.
D. NR1/NR2B NMDA receptors:

It has observed that NMDAR exhibit different kinetic and pharmacological properties depending upon the NR2 subunit present in the tetramer (Yuan et al. 2009; Gielen et al. 2009). As a result, the biophysical properties and physiological roles are fine tuned by the NR2 subunit present in the tetramer. The focus of our work is to better understand the channel kinetics of NR2B containing NMDAR.

The four NR2 subunit isoforms (A-D) are expressed differentially as compared to the obligatory NR1 subunits which are ubiquitously expressed. In the embryonic brain, NR2B and NR2D subunits predominate. NR2B subunits are mainly expressed in the cortex, hippocampus, septum, striatum and thalamic nuclei. However there is a developmental switch in the NR2B expression pattern. The adult brain has NR2B expression mainly in the forebrain areas (Watanabe et al., 1992; Akazawa et al., 1994). NR2B containing NMDA receptor activation has been proposed to induce long term depression (LTD) (liu et al., 2004; Massey et al. 2004).

There is emerging evidence that each of the NDMA subtypes play different role in the neuronal physiology and various pathological conditions. Modulation of specific NMDAR subtype for treatment of CNS disorders would enable us to avoid the side effects arising due to the indiscriminate blockade of NMDAR. NR2B-selective antagonists could be therapeutically used in the treatment of disorders like chronic pain, Parkinson’s disease, Alzheimer’s disease, cerebral ischemia and major depression (Chizh et al., 2001; Parsons et al., 2007; Loschmann et al., 2004; Steece-Collier et al., 2000; Zarate et al., 2006; Berman et al., 2000). Newer generations of selective negative modulators of NR1/NR2B receptor function have entered clinical trials and
offer a promising potential for the treatment of a wide range of CNS disorders. Despite the fact that these drugs have entered clinical trials, their mechanism of action is still unclear.

The main goal of our research is to study the mechanism of action of Ifenprodil, a well known negative allosteric modulator of NR1/NR2B receptor function.

E. Amino Terminal Domain (ATD)

The amino terminal domain (ATD) is composed of approximately the first 350-400 amino acid residues of the protein. Structurally ATD appears to form a bilobate clamshell which interacts with various extracellular allosteric modulators such as zinc for NR2A (Paoletti et al., 1997; Choi and Lipton, 1999; Fayyazuddin et al., 2000; Low et al., 2000; Choi et al., 2001), and ifenprodil for NR2B (Perin-Dureau et al., 2002; Malherbe et al., 2003; Wong et al., 2005; Ng et al., 2008).

It is a well established fact that the amino terminal domain (ATD) of the NR2 subunit controls pharmacological and kinetic properties of NMDAR (Yuan et al. 2009; Gielen et al. 2009). The agonist potency, deactivation time course, open probability ($P_{\text{Open}}$), mean open and shut duration are differentially regulated by the ATD of various NR2 subunits. (Yuan et al. 2009, Gielen et al. 2009) Therefore, understanding how the ATD affects the function of NR2 subunits will provide valuable insight into the mechanisms of channel activation for NMDA receptors.
Figure 4: Influence of the ATD and the short linker connecting the ATD and LBD on the NMDAR open probability ($P_{open}$). When the ATD+L regions of NR2A and NR2B subunits were swapped, the respective single channel $P_{open}$ were also interchanged, tending towards the wt open probabilities of NR2B and NR2A containing NMDA receptor respectively. (Figure obtained from Gielen et al., 2009)

The ATD and the linker region joining the ATD and LBD have been reported to control the open probability of the channels (Figure 4). When the ATD and linker segment connecting the ATD and LBD were swapped the open probability of NR2A and NR2B containing NMDAR were also swapped (Gielen et al., 2009). Hence the ATD-LBD linker might be crucial in transferring the conformational strain due to ligand binding in the ATD to the LBD. The open and the closed conformations of the ‘semi autonomous’ amino terminal domain (ATD + L) has been reported to control the $P_{open}$ of the NMDA receptors, as described by the model in Figure 5.
Figure 5: Model depicting NMDA receptor activity controlled by the ATD of NR2 subunits: The ATD can assume at least two conformations, a ligand-free open state and a ligand-bound closed state. The ATD spontaneously oscillate between an open-cleft conformation and a closed-cleft conformation, favoring pore opening and closure, respectively. Allosteric inhibitors of NMDARs such as NR2-ATD ligands (zinc, ifenprodil, etc.) bind to the bilobate ATD cleft and stabilize the closed state favoring pore closure. Positive allosteric modulators, on the other hand, lead to an open ATD conformation enhancing receptor activity. (Figure obtained from Gielen et al. 2009)
F. Modulators of NMDAR:

One of the characteristic features of NMDAR is that their ion channel activity is allosterically regulated by binding of modulators to the amino terminal domain (ATD). NMDAR modulators like ifenprodil, zinc, pregnanolone and protons have been well studied in the past (Popescu and Auerbach, 2003; Banke and Traynelis 2003; Auerbach and Zhou, 2005; Banke et al. 2005; Schorge et al., 2005; Erreger et al. 2005; Erreger et al. 2008; Kussius et al. 2009 a and b). These modulators can be used as ‘lead molecules’ to design better chemical moieties to inhibit NMDAR mediated neurotoxicity in pathological conditions.

1. Ifenprodil

![Diagram of Ifenprodil and its site of action on NR2B subunit of NMDAR]

Figure 6: (A) The chemical structure of ifenprodil. (B) Site of action of ifenprodil: It binds to the ATD of NR2 subunit of the receptor.
Ifenprodil is a prototype of the ‘prodil’ family of negative allosteric modulators of NR2B containing NMDARs (Mony et al., 2009). Ifenprodil is a voltage-independent, non-competitive antagonist with approximately 400-fold higher affinity for NR1/NR2B receptors over NR1/NR2A NMDAR (Williams, 1993). Chemically, ifenprodil is a phenylethanolamine (figure 4A) and it acts upon the ATD of NR2B subunit (figure 4B).

Ifenprodil blocks the receptors in a use dependent manner. It binds to the activated and desensitized states of the receptor with a higher affinity than the unliganded states (Kew et al. 1996). Previous work suggests that ifenprodil stabilizes an agonist-bound state of the receptor that has low open probability. The mechanism for this effect is still unclear. However the inhibitory effects of ifenprodil were found to be due to a reduction in the duration of long openings only. (Legendre and Westbrook, 1991, Mott et al. 1998)

![Figure 7](image)

Figure 7. Ifenprodil potentiates inward currents evoked by low NMDA concentrations. At higher concentrations, ifenprodil inhibits the NMDA mediated currents in NR1/NR2B receptors. (Figure obtained from Kew et al., 1996)
Ifenprodil binding to NMDAR results in a 3-6 fold higher affinity for glutamate and reduced affinity for glycine. (Ransom, 1991; Legendre and Westbrook, 1991). Previous studies have demonstrated that at low concentrations of NMDA, ifenprodil has a potentiating effect on the receptor currents. As the concentration of NMDA is increased, the effect of ifenprodil on NMDA-evoked currents changed from one of potentiation to one of inhibition. (Figure 7)

Originally, ifenprodil was developed as a vasodilator. It was later reported that it had neuroprotective effects too (Gotti et al., 1988). However, owing to its side effects, it is not clinically used. Numerous analogs of ifenprodil have entered clinical trials; traxoprodil, besonprodil, radiprodil to name a few. These agents have improved potency, selectivity and oral bioavailability in comparison with ifenprodil. However, the mechanism of action of these agents is still unclear.

The ATD of NR2 subunits, as previously mentioned, have been shown to differentially control the kinetics and pharmacology of the NMDAR. Zinc and Ifenprodil are the only known negative modulators of NMDARs that act upon the ATD. It has been proposed that NR2A-ATD closure following the binding of zinc pulls the NR1/NR2A LBD apart, enhancing proton binding and pore closure (Low et al. 2000). However, in case of Ifenprodil, the mechanism of strain transduction from the ATD to the channel closure still remains to be elucidated. We have used single channel recordings to investigate ifenprodil’s mechanism of action on the NR1/NR2B receptors.
2. Zinc:

Zinc ions (Zn2+) are present in the presynaptic vesicles at glutamatergic synapses. Zinc is released in an activity-dependent manner. Under physiological conditions, extracellular zinc might play an important role in modulation of NMDA receptors. Zinc binds to the ATD of NMDA receptors. The ATDs of both NR2A and NR2B subunits bind zinc with high (nM) and low (mM) affinity, respectively (Choi and Lipton, 1999; Paoletti et al., 2000; Low et al., 2000; Karakas et al., 2009).

Figure 8: Binding of zinc to isolated NR2B ATD. Both zinc and ifenprodil (a phenylethanolamine) bind to residues in both the lobes, D1 and D2, of the ATD. The zinc binding sites are different from those of ifenprodil. (Figure obtained from Karakas et al., 2009)
Rachline et al., 2005) (Figure 8). Previous research suggests that zinc reduces the mean duration as well as the channel open probability of NR1/NR2A receptors. Similar to ifenprodil, zinc inhibits NMDA receptors via enhancement of proton inhibition. (Erreger et al., 2008). The detailed mechanism of action of zinc ions is described by the model in Figure 9.

The model describes zinc inhibition using three distinct domains of the receptor:

1. The N-terminal domain which is the locus for zinc binding,

2. The agonist-binding domain and

3. The transmembrane region.

First, binding of zinc to the central cleft of NR2A NTDs results in the domain closure due to stabilization of a closed-cleft configuration. Next, NTD closure exerts tension on the linker that connects the NTDs to the ABDs that leads to the destabilization of the ABD dimer assembly and enhancement of the affinity of the proton site. Lastly, the strain on the linkers connected to the transmembrane segments is relieved by pulling apart of the ABD protomers and proton binding, thereby allowing the channel to close in the agonist bound state.
Figure 9: Proposed model for mechanism of inhibition of zinc on NR1/NR2A receptors: The LBD dimer stability is crucial for the NMDAR activity. Binding of zinc to the NR2A ATD destabilized the LBD leading to channel closure (Figure obtained from Low et al. 2000).
3. **Pregnanolone:**

![Chemical structure of Pregnanolone](image)

Figure 10: The chemical structure of Pregnanolone.

Pregnanolone sulfate (PAS), a neuroprotective steroid, inhibits NMDAR *in vitro*. The NR2A containing NMDAR might be involved in the inhibitory action of PAS. However, it was recently proposed that PAS might be a use dependent allosteric inhibitor of NR1/NR2B receptors (Petrovic et al. 2005). Pregnanolone sulfate exhibits its neuroprotective effects by driving the NMDAR channel kinetics into the desensitized conformations. It decreases the mean open probability of the NR1/NR2A receptors with no effect on the open time duration (Kussius et al. 2009).

4. **Protons**

The negative modulation of NMDAR by protons has been well studied in the past. Protons inhibit NMDAR under physiological conditions. Approximately half of NR1/NR2B receptors are under tonic inhibition of protons in normal conditions. Highest pH sensitivity is exhibited by NR1a/NR2B and NR1a/NR2D receptors with an IC$_{50}$ of ~pH 7.4 (Traynelis *et al.*, 1995). This indicates that the amount of current flowing through these receptors can be considerably altered by small changes in the extracellular pH.
Under pathological conditions like ischemia, there is accumulation of extracellular glutamate which over stimulates NMDA receptors leading to the death of neurons. Drugs like ifenprodil would preferentially block NMDARs which are continuously or repetitively activated during the pathological conditions. The physiologically activated NMDARs would be comparatively unaffected by ifenprodil. Ifenprodil inhibits NMDA current by shifting the pKa of the proton sensor on the NMDA receptor to more alkaline values (Pakh and Williams, 1997; Mott et al., 1998). Under pathological conditions like ischemia, wherein there is fall in pH levels, the antagonist efficacy of ifenprodil will be further enhanced. As a result, inhibition of the NR2B-containing NMDAR by ifenprodil is mediated through an increase in proton inhibition (Pahk and Williams, 1997; Mott et al., 1998, Mony et al 2008).

Figure 11: Protonation of NR1/NR2B receptors alters the shut time histograms: The third shut-time component (Tau 3) was found to be slowed when the channels were exposed to a brief pulse of glutamate and glycine at pH 7.9 as compared to pH 6.9 (Figure obtained from Banke et al., 2005)
Previous studies have reported that protons reduce the single channel open probability but do not affect the mean open time of NR1/NR2B receptors. (Banke et al., 2005). Protonation of key residues in the NMDA receptor structure increases the proportion of receptors that enter a nonconducting conformation. Further analysis of the dwell time histograms suggested that only the third shut-time component (Tau 3) appears to be slowed by the action of protons on NR1/NR2B receptors (Figure 11). Figure 12 describes a kinetic model proposed by Banke et al., 2005 to describe the action of protons on NMDA. The model proposes that protons can bind to the channel during any of the activation steps other than the opening steps. However, the protonated receptors in the parallel scheme cannot open. Such parallel activation schemes have also been previously proposed for ifenprodil bound receptors (Kew et al., 1996).

![Figure 12](image)

Figure 12: Physical model describing the mechanism of proton inhibition of NMDAR. The scheme illustrates a physical model of NMDA receptor gating in which the receptor can bind a proton anywhere along the reaction pathway other than the preopening steps. All the rates of the protonated receptor (grey scheme) are identical to the corresponding rates in the unprotonated
state (black scheme). The protonated receptors only differ from unprotonated receptors in that they cannot open. (Figure obtained from Banke et al., 2005)

Figure 13: Co-localization of residues that influence proton sensitivity: Red and blue circles indicate positions of residues at which mutations alter the proton IC$_{50}$ by 3 folds or more. These residues are mainly localized between the linkers connecting LBD and TMD. (The ATD is not shown in the structure). (Figure obtained from Low et al. 2003)

Previous experiments suggest that residues at which mutations perturb proton sensitivity are localized to the regions between the LBD and transmembrane helices (Low et al. 2003). There are no regions outside of M3-S2 and M4-S2 linkers that might contain a distributed and diffuse proton sensor. Hence it was proposed that these linker regions communicate the effects of LBD closure to movement of transmembrane domains, are regions in which allosteric regulators
such as protons act. Furthermore, mutations in both NR1 and NR2 subunits alter pH sensitivity suggesting that the regions between M3 and M4 of both the subunits may be pH sensitive.

**G. Gating**

Gating in NMDA receptors, as considered in this dissertation, refers to the intermediate preopening and the opening states in the fully liganded receptor. Unlike many other receptors, NMDA receptor requires all the LBDs to be occupied for the channel pore to dilate.

The simplest mechanism to explain gating is the Del Castillo-Katz mechanism (Figure 14)

Figure 14: Del Castillo-Katz mechanism for receptor activation: A and R represents the agonist and the unbound receptor, respectively. AR is the agonist-receptor complex and AR* is the receptor in the open conformation. X1 to X5 represent the preopening intermediate steps in the fully liganded state. Hence when the agonist A binds to receptor R, the complex AR undergoes a number of conformational changes (X1-X5) leading to the opening of the channel pore (AR*).
H. Kinetic Models:

![Diagram of a kinetic gating model](image)

Figure 15: Components of a typical kinetic gating model: Open, closed and desensitized states along with the forward and reverse rates of transitions connecting all the receptor conformations.

In terms of gating, NMDARs exist in three conformations; namely open, closed and desensitized. Hence, NMDA gating models usually have open states, closed states and may/may not have desensitized states. A typical kinetic ‘gating’ model is pictorially depicted as follows (Figure 15): O₁, O₂ = Open states, C₁, C₂, and C₃ = closed states and D₁, D₂ = desensitized states. The numbers (rates) on the arrows represents the number of transitions, per second, that the receptor undergoes in the forward and reverse direction. (Figure 15)
I. **Rationale for the previously proposed models:**

We have considered three of the previously proposed model for NMDAR gating to evaluate the action of ifenprodil on the NMDAR. They are as follows:


![Diagram of cyclic model](image)

Figure 16: A. Shut time histograms of activation of NMDAR with partial agonists. Partial agonists at the glycine and glutamate binding sites modify only the fast and slow conformations, respectively, in NMDA receptor activation. B. ‘Cyclic’ scheme for NMDA receptor activation: The fast (f) and slow (s) rates represented by conformation changes in NR1 and NR2 subunit lead to channel pore dilation. (Figure A obtained from Banke et al., 2003 and Figure B obtained from Erreger et al., 2005)

Banke and Traynelis (2003) proposed that the NR1 and NR2 subunits activate independently. Both the subunits must have undergone conformational changes for the channel pore to open. Their studies using partial agonists indicated that partial agonist acting at the
glycine binding site (NR1 subunit) specifically changed the fast conformations of NMDA gating. Likewise, the partial agonists at glutamate binding site (NR2 subunit) changed, specifically, the slow conformations only (as shown in fig 16A). Hence, they assigned individual subunits to kinetically distinct gating steps. The faster shut rate constants were attributed to the NR1 subunit and slow ones to the NR2 subunit.

The scheme demonstrated in Figure 16B kinetically describes their model. The rates “f” (fast) and “s” (slow) that are shown in the scheme describe the conformational changes of the NR1 and NR2 subunits respectively. Hence their ‘cyclic’ model proposes that the NR1 subunits move first followed by the NR2 subunits or the NR2 subunits might move first and then the NR1. In either case, both the subunits must have undergone their respective conformational changes for the channel to open.
2. Schorge et al. (2005)’cyclic uncoupled’ model:

![Diagram of cyclic uncoupled model](image)

Figure 17: Cyclic uncoupled model proposed by Schorge et al. (2005). The two open state arise from different closed states in the same cyclic manner as described by Banke and Traynelis, (2003) model.

This model is an extension of the Banke and Traynelis 2003 ‘cyclic’ model. They reported that for NR1/NR2A NMDAR, the long openings tend to be adjacent to the short shut times and short openings tend to be next to the longer shut times. To account for this negative correlation between the open and shut interval, they connected the two open states in a manner that they arise from two different closed states, still maintaining the cyclic arrangement of the closed states (as shown in Figure 17)
3. **Kussius et al. (2009) ‘linear’ model:**

A ‘linear’ model had been proposed previously (Popescu et al., 2004), however only recently the possible structural attributes of this ‘linear’ scheme have been hypothesized (Kussius et al., 2009). As per their model, the receptor undergoes concerted movement at each step with LBD closure, transmembrane movement followed by fast oscillations in the selectivity filter leading to the channel opening.

![Figure 18: A. Linear model describing the channel activation of NMDA receptors. B. Structural conformational movements corresponding to the kinetic model of gating. (Figure obtained from Kussius et al., 2009)](image)
A cartoon representing the structural movement in the receptor corresponding to the transitions in the ‘linear’ gating model is shown in figure 18.

- **C3-C2**: Aggregate closure of the LBD of all four subunits of the fully liganded NMDA receptor. This closing of the LBD leads to conformational strain which is transferred to the other regions of the receptor like the transmembrane domains. The receptor remains in the closed state.

- **C2-C1**: Movement in the transmembrane helices due to conformational strain. The receptor remains in the closed conformation.

- **C1-O**: Fast oscillations in the selectivity filter lead to opening of the receptor channel pore.

- **C4 and C5**: Intermediate closed states; resulting from non productive closures in the LBD and/or the ATD
J. Patch clamp technique:

The primary technique used in the research project is that of Patch Clamp. This technique has been widely employed to study single or multiple ion channel currents. The basis of patch clamp technique is the formation of a tight ‘giga seal’ between the cell membrane and a fire-polished glass micropipette. The formation of a high resistance seal is instrumental for the substantial reduction in the noise to signal ratio, which is critical for single channel recordings.

There are four common configurations to the patch clamp technique:

1. Whole cell recordings
2. Cell attached recordings
3. Outside out recordings
4. Inside out recordings

In this work, we have used the cell attached mode of recording to obtain the single channel recordings.
Figure 19: Configurations of the patch clamp technique
**Cell attached recordings:**

The pipette was guided onto the cell membrane with the aid of micromanipulators. A tight seal in the order of gigaohms was established between the pipette and cell membrane with the help of suction. The patch pipette voltage was held at +70 mV and the single channel currents, if any, were amplified and recorded.

Patches were recorded under two conditions; (1) glycine + glutamate (2) Ifenprodil + glycine + glutamate. The following drug concentrations were used for the experiments.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Conc. Used</th>
<th>EC$_{50}$ values</th>
<th>Bind to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>100µM</td>
<td>0.1-1 µM</td>
<td>NR1a LBD</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1mM</td>
<td>1-4 µM</td>
<td>NR2B LBD</td>
</tr>
<tr>
<td>Ifenprodil</td>
<td>3µM</td>
<td>0.34 µM</td>
<td>NR2B ATD</td>
</tr>
</tbody>
</table>

The drugs: glutamate, glycine and Ifenprodil were present within the patch pipette itself as the recordings were obtained in the cell attached mode. The drug concentrations were chosen higher than the EC$_{50}$ values to ensure that the receptor binding pockets were always occupied.
Fabrication of the patch micropipettes:

The size, shape, glass type and the coating on the patch pipettes vary depending upon the patch clamp mode used and the abundance of the ion channels in the preparation. All these parameters were optimized to increase the probability of obtaining a patch with a single active receptor in the cell attached mode of recording.

The main steps involved in the pipette fabrication were pulling, fire-polishing and coating the pipette tips. Borosilicate glass was chosen for the recordings. The glass capillaries were pulled using a Sutter P-97 horizontal puller to obtain two patch pipettes. These pipette tips do not have smooth edges. To make the pipette tips suitable to obtain a tight seal, they were further fire-polished. The pipette tips were brought very close to a platinum wire loop while observing it under a microscope. The loop was heated by passing DC current through it. This polishes the patch pipette tip to round of the edges and to obtain a smooth surface. Fire-polishing enhances the chances of obtaining a gigaseal without rupturing the cell surface. The fire polished tip had a resistance of 20-40 MΩ.

Due to the pulling, there is dielectric loss in the glass wall which leads to noise. It is important to eliminate noise as NR1/NR2B NMDAR single channel currents are in the order of 5-10 pA. Slygard®, an insulating agent, was applied to the lower part of electrode and then quickly cured by a hot stream of air. After coating the pipette tips, they are ready for use.
Chapter 2

Effects of ifenprodil on the gating of NR1/2B NMDA receptors
A. Introduction

NMDA receptors mediate majority of the excitatory neurotransmission in the central nervous system and are involved in the processes of learning and memory. NMDA receptors assemble as tetramers and are composed of two obligatory glycine binding NR1 subunits and two glutamate binding NR2 subunits. The NR2B subunit is expressed widely in the cortex, hippocampus, septum, striatum and thalamic nuclei. NR2B subunit is also expressed in cerebellum. However, there is a developmental switch in the NR2B expression pattern and in adult brain NR2B expression is mainly restricted to forebrain areas (Watanabe et al., 1992; Akazawa et al., 1994). NR1/NR2B NMDA receptor activation has been proposed to induce long term depression (LTD) (Liu et al., 2004; Massey et al., 2004). NR2B-selective inhibitors have therapeutic potential for the treatment of disorders such as chronic pain, Alzheimer’s disease, Parkinson’s disease, cerebral ischemia and major depression (Chizh et al., 2001; Parsons et al., 2007; Loschmann et al., 2004; Steece-Collier et al., 2000; Zarate et al., 2006; Berman et al., 2000).

Zinc and ifenprodil are the only known negative modulators of NMDA receptors that act upon the amino terminal domain (ATD). It has been proposed that zinc binds to the ATD of NR2A subunit and causes the ATD clamshell to close (Paoletti et al., 1997; Choi and Lipton, 1999; Fayyazuddin et al., 2000; Low et al., 2000; Choi et al., 2001). The ATD closure exerts a strain on the LBD which leads to the widening of the LBD interface. This exposes the proton binding site leading to closure of the channel pore (Gielen et al., 2008). However, in case of ifenprodil, the mechanism of strain transduction from the ATD to the channel closure still remains to be elucidated.
One of the major hurdles in the past to study the structure-function of NMDA receptors has been the uncertainty regarding the NMDA receptor subunit arrangement. The architecture of the NMDA receptors, on the basis of GluA2 receptor X-ray structure, was suggested recently (Sobolevsky et al., 2009). NMDA receptors are arranged as dimers of dimers, each dimer composed of one NR1 and one NR2 subunit. Hence, they have a 1-2-1-2 subunit arrangement. This proposed structure provides a basis for the development of various conceptual models of NMDA receptor gating and also better structure-function understanding.

In this paper, we exploit the recently proposed subunit arrangement of the NMDA receptors and the previously proposed models of gating for NMDA receptors to understand how ifenprodil perturbs the NR1/NR2B NMDA receptor function. We recorded single channel current in cell attached as well as outside out mode and found that NR1/NR2B receptors exhibit negative correlations. Ifenprodil was found to increase the mean shut time along with a reduction in the open probability. Moreover, the single channel data fitted better to a cyclic scheme with two uncoupled open states compared to models with sequential open states. Fitting to the ‘cyclic uncoupled’ model suggests that ifenprodil modifies multiple gating rates and increases the desensitization of the receptor.
B. Materials and Methods:

*Expression of recombinant NMDA receptors:*

Human embryonic kidney (HEK) 293 cells were maintained as previously described (Banke and Traynelis, 2003). The cells were transiently transfected with Lipofectamine 2000 reagent (invitrogen). Rat NR1-1a (Genbank U11418, U08261; pCIneo vector; hereafter NR1, provided by Dr. Stephen Heinemann), NR2B (Genbank U11419, Q00960; pcDNA3.1 vector, provided by Dr. Peter Seeburg) and green fluorescent protein (GFP) in the ratio of 1:2:0.5 were used as previously described (Banke and Traynelis, 2003). Electrophysiology experiments were performed 24-48 hours after transfection.

*Electrophysiology:*

Single channel recordings in the cell attached and outside out mode were obtained from transfected HEK 293 cells. An external solution containing (in mM) 150 NaCl, 3 KCl, 10 HEPES, 0.5 CaCl₂ was used for the recordings. The pH was adjusted to 7.9 with NaOH. This solution was supplemented with 0.01 mM EDTA to chelate trace amounts of zinc. The same external solution was used for both the outside out and cell attached recordings. We obtained the recordings under two conditions: (1) 1 mM glutamate, 100 μM glycine (control patches) and (2) 1 mM glutamate, 100 μM glycine and 3 μM ifenprodil (hereafter, ifenprodil patches). For outside out recordings, agonists and ifenprodil were added to the extracellular solution and for cell attached recordings these drugs were present only in the pipette solution. The internal solution used for outside out recordings consisted of (in mM) 110 caesium gluconate, 30 CsCl₂, 5 Heps, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA, 2 Na₂ATP and 0.3 Na₂GTP (pH 7.3). Single channel recordings were obtained using an Axopatch 200B amplifier (Molecular Devices). The data was
filtered at 8 kHz (-3 dB, 8-pole Bessel) and digitized at 40 kHz with pCLAMP 10 software (Axon Instruments/Molecular Devices). The patch pipettes were held under a potential (V_m) of +70 mV for cell attached. A holding potential of –70 mV was applied for voltage-clamp recordings in outside out mode.

Data processing and kinetic modeling:

Cell attached recordings: Single channel records were idealized using QUB software (www.qub.buffalo.edu). The shut time histogram from the longest control patch (35 min) was utilized to determine the critical shut times for conditional distribution and chopping the data for kinetic modeling. The tcrits were as follows: 0.3, 1.76, 64 and 1193 ms. Several cell attached patches contained only a small number of longest lived desensitized state. We used a tcrit of 1193 ms to chop the idealized files in order to obtain homogenous data set for performing Maximum interval likelihood fitting (MIL, Qin et al. 1996). A 100 μs dead time was imposed using QUB. All the loops in the gating schemes were constrained to obey microscopic reversibility. Dwell time histograms were generated and fitted using ChanneLab (www.synaptosoft.com) with an imposed dead time of 100 μs.

To evaluate the apparent open time adjacent to brief and longer closed durations in the cell attached recordings, open time histograms were constructed from pooled data using the shut time ranges of 0.3-1.76 ms and 1.76-64 ms respectively. These shut time ranges were determined based on the critical shut times obtained for the longest control patch as mentioned above. We subsequently constructed conditional distribution of adjacent intervals to detect any potential correlation between open and shut durations in the data. The shut time ranges used were (in ms) 0.1-0.3, 0.3-1.76, 1.76-64, 64-1193 and 1193-10000.
Outside out recordings: For the outside out recordings, the idealized files were segmented with a critical shut duration (tcrit) of 100 ms as previously described for NR1/NR2B receptors (Erreger et al., 2005). Any segment with simultaneous double channel openings was discarded. This enabled us to separate the openings within the same activation burst from openings within two different activations. Idealized activations were then fitted to hidden Markov models using the MIL method.

Statistical Analysis:

All the values are expressed as mean ± SEM. Data was compared using unpaired t test for the cell attached patches and paired t test for outside out data. Values of P < 0.05 were considered significantly different.
C. RESULTS:

Allosteric modulators, zinc and ifenprodil have been known to bind to the NR2A and NR2B ATD respectively and inhibit NMDA receptor. Moreover, recent studies have established a crucial role of NR2 subunit ATD in regulating the open probability and pharmacological characteristics of the receptor (Gielen et al., 2009; Yuan et al., 2009). In light of the advances in understanding the gating of NMDA receptors, we performed single channel recordings to identify the effect of ifenprodil on NR1/NR2B receptor gating.

NR1/NR2B receptors exhibit modal gating that persists in the presence of ifenprodil

Previous studies have reported modal gating in acetylcholine receptors, potassium channels, calcium channels, sodium channels and chloride channels (Naranjo and Brehm, 1993; Howe and Ritchie, 1992; Ivanova-Nikolova et al., 1998; Catacuzzeno et al., 1999; Delcour et al., 1993). Modal gating has also been documented for NR1/NR2A receptor (Popescu and Auerbach, 2003) and more recently for NR1/NR2B receptors (Amico-Ruvio and Popescu, 2010). We tested whether ifenprodil modifies modal gating of NR1/NR2B receptors. Single channel currents were recorded in the cell attached mode from HEK293 cells expressing NR1 and NR2B subunits. Saturating concentrations of glutamate (1 mM) and glycine (100 µM) were used. We observed modal gating in all of the nine control patches with periods of high and low open probability. Modes were assigned to the recordings based upon the open probabilities ($P_O$) of activation bursts, as reported by Amico-Ruvio and Popescu (2010). There were four patches that demonstrated the high state ($P_O \sim 0.6$), eight patches that had medium ($P_O \sim 0.3$) and six patches with the low state ($P_O \sim 0.1$). Figure 1A shows the transition of the receptor from high state to the low state. In addition to distinct bursts in high and low modes, changes in mean open time
were also observed within an activation burst. Similar modal gating was observed in ifenprodil patches. We observed four patches with high state, six with the medium and all the nine demonstrating the low state of modal gating. Channels in each of the eighteen cell attached recordings switched modes both between and within activation bursts. Figure 1B shows one such recording where the channel showed a transition from the low to high gating mode within an activation burst in the presence of ifenprodil. Hence, modal behavior persisted in the presence of ifenprodil in cell attached patches.

*Ifenprodil reduces the open probability of NR1/NR2B receptors*

We next analyzed the data to address effect of ifenprodil on channel properties. Single channel data was idealized using QUB software and further analysis was performed using Channelab software. In our single channel recordings in cell attached mode, we observed that a small set of patches (n = 2 for each control and ifenprodil, Supplemental figure 1) displayed higher overall open probability. This may be due to a dominant high mode in these patches as previously reported (Amico-Ruvio and Popescu, 2010). It would not be practical to evaluate the action of ifenprodil amidst this variability as it may obscure the results. Thus we considered seven out of nine patches under each condition for comparison of mean open time, open probability and mean shut time. Although these patches had an overall low open probability (considered over the length of entire recording), they did exhibit high mode activity lasting from milliseconds to seconds.

The mean open time (±SEM) in control patches was found to be 0.92 ± 0.14 ms (n = 7). In the presence of 3 µM ifenprodil there was no significant decrease in the mean open time; 0.92 ± 0.22 ms (n = 7). This finding is in contrast to the previous finding in hippocampal neurons.
where ifenprodil was found to reduce mean open time (Legendre and Westbrook, 1991). This difference may be attributed to the mixed NR2 subunit expression in neurons compared to only NR2B expression in our system. Differences in recording solution particularly the pH may also lead to these contrasting results. Another possibility is that the presence of modal gating in NR1/NR2B receptors may lead to variability in these unpaired patches to obscure a significant effect on mean open time by ifenprodil. However, as discussed later, we did not observe a significant change in mean open time when we obtained paired outside out patches in the absence and presence of ifenprodil further confirming the lack of effect of ifenprodil on mean open time. Interestingly protons, whose actions are closely related to ifenprodil (Mott et al., 1998), similarly have no significant effect on the mean open time of NR1/NR2B receptors (Banke et al., 2005). The mean shut time was found to significantly increase from $24.15 \pm 4.8$ ms in control patches ($n = 7$) to $70.18 \pm 16.45$ ms in ifenprodil patches ($n = 7$, $p<0.05$, unpaired $t$-test). The open probability was measured over the entire length of recordings and was found to reduce from $0.0436 \pm 0.009$ in control patches ($n = 7$) to $0.0184 \pm 0.005$ in ifenprodil patches ($n=7$, $p<0.05$, unpaired $t$-test) which may be explained by increase in the mean shut time in the presence of ifenprodil. The amplitude of openings was unaffected by ifenprodil with amplitude being $7.0 \pm 0.5$ pA for control patches ($n=9$) and $6.0 \pm 0.5$ pA for ifenprodil ($n=9$) (data not shown). Thus ifenprodil inhibition of NR1/NR2B receptors appears to be solely due to the reduction in the probability of opening of the receptor.
Ifenprodil increases the occupancy of long-lived shut states

The open and shut time histograms were fitted using a Maximum likelihood method (Colquhoun and Sigworth, 1995). The global open time histogram was best fitted by the sum of three exponential components with the time constants and areas being; control, $\tau_1 = 0.06$ ms (37%), $\tau_2 = 0.66$ ms (33%) and $\tau_3 = 1.68$ ms (30%) ($n=7$, 81602 open events, low patches only) and ifenprodil, $\tau_1 = 0.06$ ms (54%), $\tau_2 = 0.6$ ms (31%) and $\tau_3 = 1.74$ ms (15%) ($n=7$ and 17891 open events, low patches only). Although the area of the $\tau_1$ and $\tau_3$ appear to be different, there was no difference in the global mean open time. A feature of modal gating is an increase in mean open time when the receptor switches from low, medium to high mode (Popescu and Auerbach, 2003; Amico-Ruvio and Popescu, 2010). Moreover, the open time histogram under each of these modes can be fitted to two components and only the longer time constant was found to shift, increasing from low to high mode (Popescu and Auerbach, 2003; Amico-Ruvio and Popescu, 2010). Thus if the channel is operating only in one mode the open time histogram can be fitted to two components. However, if three or four components are required to fully describe the data, then it would suggest that the channel flipped between modes at least once during the length of the recording. We found that at least three open components were needed to obtain a high quality fit for the single channel data further suggesting that the channel at least flipped between two modes. This is in agreement with high and low modes that we commonly observed in our recordings.

The composite shut time histogram was fitted by five exponential functions with the time constants and areas being; control, $\tau_1 = 0.08$ ms (7%), $\tau_2 = 0.98$ ms (49%), $\tau_3 = 16.9$ ms (38%), $\tau_4 = 177$ ms (5.5%) and $\tau_5 = 4122$ ms (0.5%), (81610 closed periods, low only) and ifenprodil, $\tau_1 = 0.11$ ms (8%), $\tau_2 = 0.89$ ms (16%), $\tau_3 = 18.9$ ms (49%), $\tau_4 = 109$ ms (23%) and $\tau_5 = 1003$ ms.
ms (4%) (18521 closed periods, low only). The global shut time fit clearly indicates a difference in area of $\tau_2$ which can be visually observed in Figure 2. Individual shut time histograms were subsequently fitted to statistically compare the difference in time constants and area. Several patches that we obtained did not have enough events in the range of the longest time constant and could be fitted with four components. Therefore, in order to maintain consistency in fitting time constants across different patches we used a $t_{crit}$ of 1193 ms (see Methods) to chop the data and fitted all the shut time histograms obtained subsequently to four exponential functions. The mean ± SEM time constants and areas are presented in Table 2. We found a significant decrease in the area of $\tau_2$ and an increase in the area of $\tau_3$ and $\tau_4$ but no change in the time constants themselves (Table 2) suggesting that ifenprodil primarily increases the probability that the receptor occupies one of the long-lived shut states.

**NR1/NR2B receptors display conditional distribution**

In case of nicotinic and glycine receptors, it has been found that the long openings tend to be adjacent to the short shut times and short openings tend to be next to the longer shut times (Colquhoun and Sakmann, 1985; Hatton et al., 2003; Beato et al., 2002, 2004; Burzomato et al., 2004). Similar negative correlations have previously been reported for recombinant NR1/NR2A NMDA receptors (Gibb and Colquhoun, 1991; Schorge et al., 2005, Wyllie et al., 2006, Erreger and Traynelis, 2008) which are independent of glycine or glutamate concentration (Schorge et al., 2005). We performed a Runs test to evaluate correlations in NR1/NR2B receptors recorded in cell attached mode. It has been previously reported that the conditional distribution is dependent upon the detection of short shut times in the recordings and the imposed dead time (Schorge et al., 2005). Thus to eliminate any false negatives in our ability to detect correlations we only used patches with more than 5000 events to be certain that enough short shut times were
present. We found significant correlation between the open and shut durations in all six control patches that had > 5000 events, with the Z-test statistics ranging from -12.9 to -2. Two ifenprodil patches that had more than 5000 events also showed significant Z-statistics ranging from -7.2 to -2. Thus presence of negative correlation is a feature common to NR1/NR2A and NR1/NR2B receptors.

To further ascertain the existence of correlations in the data, we constructed conditional distribution of adjacent intervals. The shut time fitting of the longest 35 minute control patch was used to determine the tcrits as previously described in Methods (Jackson et al. 1983). The critical times were 0.3, 1.76, 64 and 1193 ms. Hence we used 0.1-0.3 ms, 0.3-1.76 ms, 1.76-64 ms, 64-1193 ms and 1193-10000 ms as the shut time ranges to examine the conditional distribution. The average ± SEM of apparent mean open time for all the patches in a given shut time range is presented in Figure 3. We found that the duration of apparent mean open time was dependent on the shut time range and an inverse relationship was clearly observed both in control and ifenprodil patches. Open time histograms were constructed from pooled data for apparent open time adjacent to a brief shut time range (0.3-1.76 ms, dark histogram) and adjacent to a longer shut time range (1.76-64 ms, grey histogram). The histograms were fitted to three exponential components. The distribution of open times adjacent to brief and prolonged close durations have similar fitted time constants (Figure 3). However, the area of the shorter time constant was greater for the longer shut time range suggesting that the duration of apparent mean open time was dependent on the shut time range. The presence of strong negative correlations in the data suggests that the gating models describing the NR1/NR2B receptor single channel activity must have two open states that are most likely connected to two separate gateway states (Schorge et al., 2005).
In recent years a number of conceptual models for NMDA receptor gating have been proposed (Popescu and Auerbach, 2003; Banke and Traynelis, 2003; Erreger et al., 2005; Auerbach and Zhou, 2005; Schorge et al., 2005; Dravid et al., 2008; Kussius and Popescu, 2009a). The first cyclic model proposed that the receptor underwent two independent conformational changes, presumably corresponding to NR1 and NR2 subunit dimers, following which the channel pore opens (Banke and Traynelis, 2003). Scheme 2 presented in Figure 3 is based on the same idea but include an additional shut state where both conformational changes have occurred that further leads to the sequential open states. An extension of this model was suggested by Schorge et al. (2005) which took the observed correlations in NR1/NR2A receptors into consideration and connected two open states separately from two gateway states (Scheme 3). A linear model of NMDA receptor gating has also been proposed (Popescu & Auerbach, 2003; Erreger et al., 2005) which is thought to signify the concerted motion of the entire receptor during the activation pathway (Kussius and Popescu, 2009) (Scheme 1). All the models contain two desensitized states to account for the long-lived shut states that we observed in our patches similar to those previously used for NR1/NR2C, NR1/NR2A and NR1/NR2B receptors (Dravid et al., 2008; Dravid et al., 2010; Kussius and Popescu, 2009a; Amico-Ruvio and Popescu, 2010).

We fitted the three previously proposed gating models for NMDA receptors (Scheme 1, 2 and 3; Figure 3) to the single channel data to evaluate the effects of ifenprodil on NR1/NR2B receptor gating. The concentrations of glutamate and glycine used were ~100–1000 fold more than EC50 to ensure rapid agonist rebinding so that the closed times due to rebinding are exceptionally brief. Thus all the states represent receptor conformations with fully bound glutamate and glycine. It must be noted that the total number of shut states in the three schemes...
is not the same. Unlike Schemes 2 and 3, which have six shut states, Scheme 1 has only five shut states. However in all the three schemes, the data has been fitted to describe the same number of rate constants since microscopic reversibility was imposed for the loops in Schemes 2 and 3. As a first step to establish which of the three models (Scheme 1, 2 and 3) fitted the data better, we performed maximum interval likelihood (MIL) fitting of the idealized data from the longest (35 minute) patch to the models. As seen in figure 3, the Log likelihood (LL) values were 305611.5, 305611 and 306350 for Scheme 1, 2 and 3 respectively. Thus based on the LL values, we found that the model proposed by Schorge et al. (2005) (Scheme 3) yielded a better quality fit compared to the other two models. Based upon the LL criterion, we have considered only two models, the ‘linear’ model with the sequential open states (Scheme 1) and the ‘cyclic uncoupled’ model with the uncoupled open states (Scheme 3) for further analysis.

We next tested whether ‘cyclic uncoupled’ model had a consistently better LL compared to Scheme 1 across all the patches. As mentioned previously several patches that we obtained did not have enough events for the longest time constant and could be fitted with four components. Therefore, in order to maintain consistency in MIL fits across different patches we used a tcrit of 1193 (see Methods) to chop the data. To account for this loss of information, we did not include the long-lived desensitized (D1) state in subsequent MIL fits. Upon performing MIL fits to all the patches that had been segmented using the tcrit, we found that the LL values were consistently higher for the ‘cyclic uncoupled’ compared to the ‘linear’ model’. A difference of 10 LL units was considered to be a better fit. The MIL fits were better for seven out of nine control patches and six out of eight ifenprodil patches with the ‘cyclic uncoupled’ model. The ‘linear’ model proved to be a better only for one control and one ifenprodil patch. Both the models fitted the data equally well for one control and one ifenprodil patch.
Ifenprodil affects multiple gating steps

Next we evaluated the effect of ifenprodil in the framework of the ‘linear’ and ‘cyclic uncoupled’ model of NR1/NR2B receptor gating. As mentioned previously a tcrit of 1193 was applied to the patches for MIL fits and only one desensitized state (D2) was included in the models to account for this loss of long-lived shut state. The mean ± SEM rate constants obtained from MIL fitting of the idealized data are presented in the Table 1 and Figure 4. First, we consider the MIL fits to cyclic uncoupled model (Scheme 3). For Scheme 3 we found that both the forward ($k_1^+$) and reverse ($k_1^-$) rates of a fast gating step were reduced whereas the reverse rate ($k_2^-$) of a slow gating step was increased two-fold. In addition the rate of entry into the desensitized state ($d_2^+$) was increased. The channel opening rates were unaffected by ifenprodil, in agreement with the lack of effect of ifenprodil on the mean open time in our data. When the idealized data was fitted to Scheme 1, we found that only the forward rate of a fast preopening step was reduced and all other rate constants were unaffected. A representative patch with the fitted dwell time histogram is shown in figure 4A.

The rates derived from the MIL fit for the ‘linear’ and ‘cyclic uncoupled’ model were used in a Monte Carlo simulation of a single active channel activated by a maximal agonist concentration. To estimate the rates of the longest shut time constant we utilized patches which had sufficient events in the range of the longest time constant. These patches were individually fitted and the averaged rates were used for the longest time constant (D1, Figure 3, Schemes 1 and 3). The simulations accurately predicted a reduction in the open probability of NR1/NR2B receptors in presence of ifenprodil. Monte Carlo simulations using the cyclic uncoupled model predicted that the ifenprodil might reduce the MOT from 1.066 (control) to 1.014. The open probability might be reduced from 0.03 (control) to 0.008 in the presence of ifenprodil.
Simulations performed using the ‘linear’ model predicted that the MOT to be as follows: control, 0.961 and ifenprodil, 1.029. The open probability might be reduced from 0.039 (control) to 0.011 in the presence of ifenprodil. A dead time of 150 µs was imposed.

Effects of ifenprodil in excised outside out recordings

Although recordings in cell attached mode have the advantage of keeping the intracellular milieu intact, paired recordings under outside out conditions may provide a better comparison between control and ifenprodil conditions. We therefore tested whether the effects of ifenprodil on NR1/NR2B kinetics observed in the cell attached recordings were conserved in outside out recording condition. Modal gating has previously been reported for NR1/NR2A and NR1/NR2B receptors in cell attached mode (Popescu and Auerbach, 2003; Kussius and Popescu, 2009; Amico-Ruvio and Popescu, 2010) and observed for outside out patches (unpublished observations). We found that NR1/NR2B receptors exhibit modal behavior in outside out recordings. Figure 5A demonstrates one such activation burst where the receptor switches between different modes. Moreover similar to cell attached patches, modal gating persisted in the presence of ifenprodil. The receptor transitioned between modes with changes in the mean open time and open probability within and between activation bursts in the presence of ifenprodil.

We have utilized recordings with single and multiple channels for analysis of channel properties owing to the lack of long paired recordings with a single active channel. Out of the six paired patches used in our analysis, two were single channel recordings and the remainder had two channels. The patches containing two active receptors were chopped using a tcrit of 100ms to obtain activation bursts with one active receptor (see Methods for details). The activation bursts obtained with a tcrit of 100 ms were utilized to evaluate the effects of ifenprodil on
channel activity. A modest but statistically insignificant reduction in the mean open time and channel open probability by ifenprodil was detected (Figure 5C and 5D). The amplitude of openings, similar to cell attached patches, was found to be unaffected by ifenprodil (data not shown). However, the mean shut time was found to be significantly different (Paired t test, P<0.05, Figure 5E). Thus the negative modulation of NR1/NR2B receptors by ifenprodil is due to the increase in the shut durations of the receptor, in agreement with our cell attached results that ifenprodil increases the occupancy of the receptor in the long lived shut conformations.

Global histograms were obtained by pooling all events from patches containing one active receptor and events chopped data (100 ms tcrit) from patches containing two active receptors. The global open time histogram was best fitted by a sum of three exponential components with the time constants and areas being; control, \( \tau_1 = 0.09 \text{ ms} \) (26\%), \( \tau_2 = 2.16 \text{ ms} \) (48\%) and \( \tau_3 = 5.77 \text{ ms} \) (26\%) (n=6, 46788 open events) and ifenprodil, \( \tau_1 = 0.08 \text{ ms} \) (38\%), \( \tau_2 = 1.49 \text{ ms} \) (42\%) and \( \tau_3 = 4.29 \text{ ms} \) (20\%) (n=6, 23802 open events). We found that at least three open components were needed to obtain a high quality fit for the single channel data suggesting that the receptor flipped between modes, in agreement with periods of high and low modes during the recordings (Figure 5A). The composite shut time histogram was fitted by a sum of five exponential functions with the time constants and areas being; control, \( \tau_1 = 0.05 \text{ ms} \) (10\%), \( \tau_2 = 0.61 \text{ ms} \) (29\%), \( \tau_3 = 11.9 \text{ ms} \) (43\%), \( \tau_4 = 65.9 \text{ ms} \) (15\%) and \( \tau_5 = 573 \text{ ms} \) (3\%) (46796 closed periods) and ifenprodil, \( \tau_1 = 0.17 \text{ ms} \) (11\%), \( \tau_2 = 1.24 \text{ ms} \) (19\%), \( \tau_3 = 22.3 \text{ ms} \) (44\%), \( \tau_4 = 202 \text{ ms} \) (22\%) and \( \tau_5 = 1107 \text{ ms} \) (4\%) (23743 closed periods). Similar to cell attached recordings, the global shut time fit depicts a reduction in the area of \( \tau_2 \) with no significant change in the time constants.
D. DISCUSSION

NMDA receptors exhibit complex single channel kinetics which have been particularly challenging to model. Nonetheless, a number of conceptual gating models have been proposed and the actions of partial agonists and allosteric inhibitors of NMDA receptors such as proton, zinc and pregnanolone have been studied in this proposed framework (Popescu and Auerbach, 2003; Banke and Traynelis 2003; Auerbach and Zhou, 2005; Banke et al. 2005; Schorge et al., 2005; Erreger et al. 2005; Erreger et al. 2008; Kussius et al. 2009 a and b). An additional advantage of studying actions of partial agonists and inhibitors is that it may provide information to resolve the complex NMDA receptor kinetics. In the current study, we used ifenprodil, a well known negative allosteric inhibitor of NR1/NR2B receptors, to investigate the potential influence of NR2B ATD on NMDA receptor gating.

There are five significant findings from our investigation: First, the inhibition of NR1/NR2B receptors by ifenprodil can solely be attributed to an increased occupancy of long lived shut time which leads to reduction in the open probability of the receptor with no significant influence on mean open time and amplitude. Second, modal gating appears to be an integral part of the NDMA receptor gating machinery and was observed both in cell attached and outside out patches which further persisted in the presence of ifenprodil. Third, similar to NR1/NR2A receptors negative correlations between the open and shut intervals are observed in NR1/NR2B receptors both in control and ifenprodil patches. These findings suggest that a gating model that incorporates both features; modal gating and conditional distribution, is essential to accurately describe NR1/NR2B channel activation pathway. Fourth, based on the log likelihood criterion, the cyclic model with uncoupled open states, as proposed by Schorge et al. (2005), described our single channel cell attached data in a better manner than the linear model.
Lastly, MIL fit to a ‘cyclic uncoupled’ scheme suggests that ifenprodil and hence the ATD, influences multiple gating steps. In contrast, only one gating step in a linear scheme was significantly modified by ifenprodil. Results from this study also reinforce the idea that NMDA receptor gating may involve both subunit independent and concerted movements of the NR1 and NR2 subunits.

*Allosteric modulation by ifenprodil*

Ifenprodil was identified as a NR1/NR2B selective inhibitor (Williams, 1993) that binds to the NR2B ATD (Perin-Dureau et al., 2002; Malherbe et al., 2003; Wong et al., 2005; Ng et al., 2008). The effect of ifenprodil on macroscopic currents in neurons and heterologous expression system suggests that ifenprodil inhibition is use-dependent. It preferentially binds the receptor in open and desensitized conformation (Kew et al., 1996). This is in agreement to MIL fit with *Scheme 3* where ifenprodil increased the rate of entry of the receptor to a fast desensitized state. At the single channel level, ifenprodil has been shown to reduce the duration of the long openings and open probability of the receptor (Legendre and Westbrook, 1991). We did not find a significant decrease in MOT by ifenprodil which may be explained, among other differences in the preparation and recording conditions, by difference in pH (7.9) of our recording solution. Protons are known to tonically inhibit NMDA receptors. At physiological pH, about 50% of the NMDA receptors are inhibited (Traynelis *et al.*, 1995; Mony et al. 2009). Additionally previous work suggests that both zinc and ifenprodil make NMDA receptor more sensitive to proton block (Pahk and Williams, 1997; Mott et al., 1998; Choi & Lipton, 1999; Low *et al.* 2000; Zheng *et al.* 2001; Erreger and Traynelis, 2005; Erreger et al. 2008). Thus one possibility is that since ifenprodil inhibition is greater at lower pH, a difference in pH of recording condition may affect the influence of ifenprodil on MOT. Interestingly, proton inhibition of NR2B containing NMDA
has striking similarities with that of ifenprodil blockade of the NR1/NR2B receptors. Protons have been reported to affect only the open probability of the NR1/NR2B receptors with no significant reduction in the mean open time (Banke et al., 2005) similar to our findings with ifenprodil. In contrast, allosteric inhibition of NR1/NR2A receptors by proton and zinc involves reduction in mean open time indicating that unique differences do exist in how ATD controls gating among various NR2 subunits (Erreger and Traynelis, 2008).

**NMDA receptor single channel kinetics: Modal gating and conditional distribution**

Previous studies reporting single channel characteristics of NR1/NR2B receptors in outside out and cell attached patches suggest that the mean open time and open probability is lower in excised patches compared to cell attached patches (Banke and Traynelis, 2003; Banke et al., 2005; Erreger et al., 2005). However, differences in extracellular recording solutions used in these studies, including calcium, EDTA concentrations and pH, limit direct comparison. We studied NR1/NR2B receptor characteristics in cell attached and excised patches under the same extracellular conditions and found modal gating in both types of patches which also persisted in the presence of ifenprodil. Interestingly, the mean open time and open probability was higher for excised patches compared to cell attached patches suggesting that in excised condition the receptor is under a conformation that allows a more stable open state. However, despite this difference in mean open time the overall distribution of the shut time histograms in outside out and cell attached patches were very similar, especially the shut time histograms in the presence of ifenprodil. These results suggest that different modes of recording may affect the stability of the open state but may not influence the gating steps leading to channel pore dilation.
Negative correlations have been previously described for NR1/NR2A receptors (Gibb and Colquhoun, 1991; Schorge et al., 2005; Wyllie et al, 2006; Erreger and Traynelis, 2008) and have been accounted by the gating model proposed by Schorge et al. (2005). We found that the duration of a sojourn in an open state was negatively dependent on the adjacent shut interval for NR1/NR2B receptor in the cell attached patches. Negative correlation was also maintained in the presence of ifenprodil. Presence of correlations suggests that the two open states are most likely connected to two separate gateway states (Schorge et al., 2005). Interestingly, we consistently found that the ‘cyclic uncoupled’ model yielded a superior quality fit as interpreted by higher LL compared to linear and the cyclic model with sequential openings. The simplest explanation for this observation will be that the uncoupling of open states accommodates the feature of negative correlations and therefore better predicts the receptor activation compared to sequential open states. The two open states in the cyclic model in Scheme 3 arise from two different shut states and have different stabilities, with O1 shorter in duration compared to O2. The opening rate for long-lived O2 state was found to be between 25000 and 35000 sec\(^{-1}\) for the control and ifenprodil patches (fig 4) which is similar to the rate previously reported by Schorge et al. (2005). The two open states most likely represent different receptor conformations but must have the same channel pore size since only one conductance level was observed in the recording.

**NR1/NR2B receptor gating and influence of ifenprodil**

Banke and Traynelis (2003) proposed that the individual subunits undergo independent conformational changes. The faster shut rate constants were attributed to the NR1 subunit and slow ones to the NR2 subunit. Schorge et al. proposed the same cyclic model with the uncoupled open states in 2005. As per their proposal, our results (Figure 3B) suggest that the NR1 step of gating was slower in both forward (k1) and reverse (k1-) direction when ifenprodil was bound to
the ATD of the NR2 subunit. Additionally, the reverse rate (k2-) of the NR2 step was enhanced by ifenprodil. Thus, in presence of ifenprodil, the receptor kinetics might be driven into the C1, C2 and C3 shut conformations. This would mean that the receptor will open more in the short opening (O1) reaction pathway or desensitize more often than in control conditions. This effect is reflected in the ifenprodil open time histograms where the area of the fast open time constant increased in both the cell attached and outside out data. So also, the area of the long open time constant was decreased in the presence of ifenprodil. This is in agreement with previous reports, where ifenprodil was found to decrease the duration of long openings only (Legendre and Westbrook, 1991). Thus if we consider receptor activation in the framework of subunit independent steps, our results suggest that actions of ifenprodil are not limited to the NR2B subunit rather it has far reaching effects on both, the NR1 and NR2 subunit, as a negative allosteric modulator of the receptor.

Kussius and Popescu (2009) assigned different conformational states to the linear model of NMDA receptor gating. They proposed that the receptor undergoes concerted movement at each step with LBD closure and transmembrane movement followed by fast oscillations in the selectivity filter. Thus based on the fitting of data to the linear model, the rates suggested that binding of ifenprodil selectively slows the movement of the transmembrane domains of the receptor since only the k2 forward rate was significantly reduced. The desensitization rates were not modified in presence of ifenprodil in the linear model.
**Structural basis of NR1/NR2B receptor gating**

It has been long known that binding of the ligand and the subsequent conformational changes in the receptor architecture leads to opening of the NDMA receptor ion channel pore. However, the exact nature of these conformational changes is still poorly understood. The recently solved architecture and symmetry of the iGluRs, based on the GluA2 structure, provides us with useful information to better understand the NMDA receptor gating (Sobolevsky et al., 2009). Activation of the iGluRs originates in the LBDs. The agonist binding induces the clamshell closure which pulls the M3 domains apart in an iris like fashion leading to the channel opening. Based on the crystal structure, it is predicted that agonist binding will have discrete consequences on the NR1 and NR2 subunits. The NR1 subunits are proximal to the channel pore and the NR2 subunits are distal to the pore. The extent of conformational movement of the distal NR2 subunits is considerably different and greater as compared to the movements of the proximal NR1 subunits. Hence NR2 subunit dimer appears to be positioned such as to have a greater role in channel activation as evident by control of NMDA receptor kinetics by NR2 ATD (Sobolevksy et al., 2009; Yuan et al., 2009; Gielen et al., 2009). Another important feature of NMDA channel pore is the staggering of subunits. It has been demonstrated that the NR1 and NR2C subunits are positioned differently relative to the central and vertical axes of the channel pore (Sobolevksy et al. 2007, but see Chang and Kuo, 2008). Thus the homologous domains in individual NMDA receptor subunits make unequal contributions to the pore structure.

In our view, it is this differential subunit conformational displacement together with the potential vertical and axial staggering of the transmembrane domains of NR1 and NR2 subunits that are critical to the unique NMDA receptor kinetics. We suggest that the fast shut rates may indeed represent the NR1 step as the subunit has to undergo a lesser conformational
displacement. On the other hand, the NR2 subunits may have slower kinetics due to the larger conformational displacement. Since the subunits are undergoing dissimilar conformational changes, the structural explanation for the linear model may be deficient as it predicts that each subunit undergo the same amount of conformation during each of the proposed transitions.

Based on our results, the ‘cyclic uncoupled’ Schorge et al. (2005) model provides a better fit to the NR1/NR2B single channel data. Thus the ‘cyclic uncoupled’ model applies to both NR1/NR2A and NR1/NR2B receptors. However to provide a structural explanation for this model is challenging, particularly the two open states with different stabilities, The conformational changes in the transmembrane domains and the selectivity filter TM2 would be critical to explain the two open states. Further studies are required to determine the precise roles of pre M1, M3-S2 and S2-M4 linkers to appreciate the detailed conformational movements of these important structures.

Our understanding of NMDA gating models and more importantly its structural explanations is still very limited. However, when viewed under the light of the recently resolved crystal structure of the GluA2 and its extrapolation to NMDA receptors and conditional distribution; our results suggest that the cyclic model with uncoupled open states is a better model to capture some of the unique kinetic properties of the NMDA receptors.
E. Figures:

**Figure 1: Modal gating in NR1/NR2B receptors in the cell attached patches:**

A, Representative steady state single channel recording in cell attached mode from patches containing one active NR1/NR2B receptor. Openings are upward for all the traces. Upper trace: obtained at saturating concentrations of glutamate and glycine (1 mM, 100 µM respectively, Pipette potential = +70 mV, filtered at 10 kHz, digitized at 40 kHz, duration 10 sec) shows the transition from the high to low mode of NR1/NR2B NMDA receptor gating. Lower trace:
obtained in the presence of ifenprodil (3 µM) shows the transition from low to high mode of
gating. (B) Upper trace: recorded in the presence of ifenprodil shows the transition of the
receptor to different gating modes within an activation burst. The underlined sections of the trace
are expanded in the following three traces (duration 100 ms each) demonstrating high (H),
medium (M) and low (L) modal behavior of NR1/NR2B receptor.
Figure 2: Effects of ifenprodil on the NR1/NR2B receptor single channel kinetics

The single channel currents from cell attached patches containing one active NR1/NR2B receptor were idealized to generate dwell time histograms. A dead time of 100 μs was imposed on the idealized data files. A, Global open and shut dwell time histograms. The open time histogram was fitted by a sum of three exponential components: Control, n = 7, 81602 open events and ifenprodil, n = 7, 17891 events (low P_o patches only). The composite shut time histogram was fitted by a sum of five exponential functions: Control, n = 7, 81610 closed periods
and ifenprodil, n = 7, 18521 closed intervals. B, The control mean open time (± SEM) was found to be 0.92 ± 0.14 ms (n = 7). There was no significant decrease in the mean open time with ifenprodil; 0.92 ± 0.22 ms (n = 7). C, The probability of opening (± SEM, calculated individually over the length of entire recording) for control patches was found to be 0.0436 ± 0.009 (n = 7). Ifenprodil significantly reduced the probability of opening of the receptors to 0.0184 ± 0.005 (P<0.05) (n = 7). D, The mean shut time (± SEM) was found to be significantly increased from 24.15 ± 4.8 ms for control patches (n = 7) to 70.18 ± 16.45 ms in ifenprodil patches (n = 7) (P<0.05). Unpaired t test was used for comparison. * denotes P<0.05
Figure 3: Conditional distribution and conceptual gating models for NR1/NR2B receptor activation.

A. Open time histograms were constructed from pooled data for apparent open time adjacent to a brief closed duration in the range of 0.3-1.76 ms (black histogram) and adjacent to a longer closed duration in the range of 1.76-64 ms (grey histogram, control n = 9, ifenprodil n = 8). The
range was chosen based on the tcrit calculated from the longest patch (35 min) (as described in methods) which were 0.3, 1.76, 64 and 1193 ms. B. Conditional distribution of adjacent intervals: The shut time ranges used were (in ms) 0.1-0.3, 0.3-1.76, 1.76-64, 64-1193 and 1193-10000. The mean open time is expressed as % maximum of the first shut time range, 0.1-0.3 ms. A negative correlation between the open and shut intervals was found in both the control and ifenprodil patches (control n = 9, ifenprodil n = 8). C. Conceptual models for NR1/NR2B receptor activation: Three previously proposed gating models for NMDA receptors were fitted to the single channel data to evaluate the effects of ifenprodil on NR1/NR2B receptor gating. *Scheme 1* has been described by Kussius and Popescu (2009) for the activation of NR1/NR2A receptors, *Scheme 2* is similar to that proposed by Banke and Traynelis (2003) for NR1/NR2B gating and *Scheme 3* was proposed by Schorge et al. (2005) for NR1/NR2A receptor activation. All the states represent receptor conformations with fully bound glutamate and glycine. Inset: log likelihood values for the longest control patch (35 min) obtained from MIL fits to Schemes 1, 2 and 3.
Figure 4: Kinetic mechanisms describing the effects of ifenprodil on NMDA receptor activation.

A: MIL fit of single channel data with Scheme 3 is shown. There were a total of 32441 open events, 32366 shut periods for the control patch (MOT = 1.5 ms, Po = 0.056) and 3731 open events, 3487 shut periods fitted for the ifenprodil patch (MOT = 0.85, Po = 0.043) (imposed resolution of 100 µs). B. Kinetic mechanism of negative NR1/NR2B receptor modulation by ifenprodil: The idealized data was chopped with a tcrit of 1193 ms (see methods) and MIL fittings of the steady state currents to Schemes 1 and 3 without the long-lived D1 state were
performed. Rate constants are means of rates obtained by fits to individual patches. Bold numbers with asterisks denote that the rates were significantly different from that of glutamate/glycine alone (P<0.05). All rates are in sec⁻¹. Data are mean ± SEM obtained from nine control and eight ifenprodil patches. Microscopic reversibility was imposed for loops in the cyclic model.
Figure 5: Effects of ifenprodil on channel kinetics in the excised outside out recordings.

Single channel currents from excised outside out patches from HEK293 cells expressing NR1/NR2B receptors. A. Representative steady state single channel recording in outside out
mode obtained with glutamate and glycine (1mM and 100µM respectively, Holding potential = -70mv, filtered at 6 kHz, digitized at 40 kHz, duration 1.5 sec) demonstrating the transition between high, medium and low modes of gating. Openings are downwards. B. Global open and shut time histograms. The global open time histogram was fitted by a sum of three exponential functions; Control: n = 6, 46788 open events and ifenprodil: n = 6, 23802 events. The composite shut time histogram was fitted by a sum of five exponential components; Control: 46796 closed intervals and ifenprodil: 23743 intervals. A dead time of 100 μs was imposed on the idealized data files. C, D, E: Effects of ifenprodil on the NR1/NR2B receptor activity. Graphs depicting the mean open time, open probability and mean shut times within an activation burst of the six paired control and ifenprodil patches (see methods). Only the mean shut time was found to be significantly different (Paired t test, P<0.05).
Supplemental Figure 1: Variability in the cell attached and outside out steady state recordings.

A, Scatter plot demonstrating the mean open times of the individual records in the cell attached (black points, control n = 9 and ifenprodil n = 9) and outside out (grey points, control n = 8, ifenprodil n = 8) single channel recording modes. B, Scatter plot showing the NR1/NR2B NMDA receptor open probability in cell attached and outside out steady state recordings. The black data points represent the open probability of the channel considered over the entire length of individual recordings (control n = 9, ifenprodil n = 9). The grey data points represent the Po within an activation burst (tcrit 100 ms) in excised outside out recordings (control n = 8, ifenprodil n = 8).
Table 1: Hidden Markov maximum interval likelihood fitting of the steady state currents.

<table>
<thead>
<tr>
<th>Rates (s⁻¹)</th>
<th>‘Cyclic uncoupled’ Model</th>
<th>‘Linear’ model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ifenprodil</td>
</tr>
<tr>
<td>(k_{1+})</td>
<td>1385 ± 200</td>
<td>730 ± 135*</td>
</tr>
<tr>
<td>(k_{1-})</td>
<td>9540 ± 1065</td>
<td>5530 ± 1510*</td>
</tr>
<tr>
<td>(k_{2+})</td>
<td>90.5 ± 25</td>
<td>79.8 ± 11</td>
</tr>
<tr>
<td>(k_{2-})</td>
<td>360 ± 45</td>
<td>745 ± 135*</td>
</tr>
<tr>
<td>(\beta_1)</td>
<td>1950 ± 575</td>
<td>1720 ± 490</td>
</tr>
<tr>
<td>(\alpha_1)</td>
<td>24910 ± 4150</td>
<td>28060 ± 6365</td>
</tr>
<tr>
<td>(\beta_2)</td>
<td>26610 ± 3955</td>
<td>32280 ± 5370</td>
</tr>
<tr>
<td>(\alpha_2)</td>
<td>4115 ± 405</td>
<td>6650 ± 2390</td>
</tr>
<tr>
<td>(d_{2+})</td>
<td>34.6 ± 9.2</td>
<td>120 ± 34*</td>
</tr>
<tr>
<td>(d_{2-})</td>
<td>5.6 ± 0.87</td>
<td>10 ± 4</td>
</tr>
</tbody>
</table>

Idealized current records were fitted to the ‘cyclic uncoupled’ and ‘linear’ gating schemes as described in Figure 4B. All rates have units of s⁻¹. Data are mean ± SEM from nine control recordings and eight ifenprodil recordings, each containing one active channel, fitted individually. The rates were compared by paired \(t\)-test. * indicates \(p < 0.05\). The rates \(k_{1+}, k_{1-}, k_{2-}\) and \(d_{2+}\) were found to be significantly different for the cyclic uncoupled model. Only the \(k_{2+}\) rate was found to be significantly different for the linear model.
Table 2: Time constants and areas of closed and open components obtained from exponential fits.

<table>
<thead>
<tr>
<th>Time constants (ms) and areas (%)</th>
<th>Control N=7</th>
<th>Ifenprodil N=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shut time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_1 )</td>
<td>0.04 ± 0.004</td>
<td>0.06 ± 0.018</td>
</tr>
<tr>
<td>( \tau_2 )</td>
<td>0.93 ± 0.07</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td>( \tau_3 )</td>
<td>23 ± 3.4</td>
<td>31 ± 5.5</td>
</tr>
<tr>
<td>( \tau_4 )</td>
<td>249 ± 34</td>
<td>245 ± 43</td>
</tr>
<tr>
<td>( a_1 )</td>
<td>3.9 ± 0.6</td>
<td>7.2 ± 2.5</td>
</tr>
<tr>
<td>( a_2 ) *</td>
<td>51 ± 2.5</td>
<td>19 ± 3.4</td>
</tr>
<tr>
<td>( a_3 ) *</td>
<td>37 ± 2</td>
<td>52 ± 3.5</td>
</tr>
<tr>
<td>( a_4 ) *</td>
<td>6.9 ± 2</td>
<td>20.4 ± 4</td>
</tr>
<tr>
<td>Open time</td>
<td></td>
<td></td>
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<tr>
<td>( \tau_1 )</td>
<td>0.06 ± 0.013</td>
<td>0.05 ± 0.017</td>
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<tr>
<td>( \tau_2 )</td>
<td>0.9 ± 0.16</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>( a_1 ) *</td>
<td>7.8 ± 0.6</td>
<td>14 ± 2.7</td>
</tr>
<tr>
<td>( a_2 ) *</td>
<td>92.15 ± 0.6</td>
<td>85.93 ± 2.7</td>
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Comparison of the time constants (\( \tau \)) and relative contribution (area of the component) of the two open time and four shut time components obtained from individual fits to the cell attached patches. Data are mean ± SEM from seven control and seven ifenprodil recordings, each containing one active channel. The values were compared by paired \( t \)-test. * indicates \( p < 0.05 \). Patches with a dominant high mode (Supplemental figure 1) were not used for this analysis.
References:


