Effect of Ifenprodil on GluN1/GluN2B N-Methyl-D-aspartate Receptor Gating

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ABSTRACT

Ifenprodil is an allosteric inhibitor of GluN1/GluN2B N-methyl-D-aspartate receptors. Despite its widespread use as a prototype for drug development and a subtype-selective tool for physiologic experiments, its precise effect on GluN1/GluN2B gating is yet to be fully understood. Interestingly, recent crystallographic evidence identified that ifenprodil, unlike zinc, binds at the interface of the GluN1/GluN2B amino terminal domain dimer by an induced-fit mechanism. To delineate the effect of this unique binding on GluN1/GluN2B receptor gating, we recorded steady-state currents from cell-attached and outside-out patches. At pH 7.9 in cell-attached patches, ifenprodil increased the occupancy of the long-lived shut conformations, thereby reducing the open probability of the receptor with no change in the mean open time. In addition, ifenprodil selectively affected the area of shut time constants, but not the time constants themselves. Kinetic analyses suggested that ifenprodil prevents the transition of the receptor to an open state and increases its dwell time in an intrinsically occurring closed conformation or desensitized state. We found distinct differences in the action of ifenprodil at GluN1/GluN2B in comparison with previous studies on the effect of zinc on GluN1/GluN2A gating, which may arise due to their unique binding sites. Our data also uncover the potential pH-dependent action of ifenprodil on gating. At a low pH (pH 7.4), but not pH 7.9, ifenprodil reduces the mean open time of GluN1/GluN2B receptors, which may be responsible for its usefulness as a context-dependent inhibitor in conditions like ischemia and stroke, when the pH of the extracellular milieu becomes acidic.

Introduction

N-methyl-D-aspartate (NMDA) receptors mediate slow excitatory neurotransmission at central synapses and are essential for synaptic plasticity and learning and memory (Traynelis et al., 2010). NMDA receptors are tetramers composed of two obligatory glycine-binding GluN1 subunits and usually two glutamate-binding GluN2 subunits. Ifenprodil is one of the earliest discovered noncompetitive NMDA receptor inhibitors, and is 100-fold more selective for GluN2B-containing NMDA receptors (Carron et al., 1971; Williams, 1993); in addition, ifenprodil analogs may have therapeutic targeting the NMDA receptor that has progressed to clinical trials, there remains a superficial understanding of its mechanisms of allosteric inhibition.

NMDA receptors exhibit unique and complex gating, including features such as correlations and modal gating. The precise structural interpretation of the gating steps before the channel opens is not fully understood. Recent progress in the understanding of the structure of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors and its extrapolation to NMDA receptors has yielded valuable insights (Sobolevsky et al., 2009). However, it remains unclear whether the GluN1 and GluN2 subunits contribute predominately to individual gating steps before the channel pore opens. Zinc and ifenprodil are negative modulators of NMDA receptors that act upon the amino terminal domain (ATD). The mechanism by which zinc affects GluN1/GluN2A receptor gating has been well characterized (Paoletti et al., 1997; Choi and Lipton, 1999; Fayyazuddin et al., 2000; Low et al., 2000; Choi et al., 2001; Erreger and Traynelis, 2008; Amico-Ruvio et al., 2011). Ifenprodil has some features similar to zinc, in that binding of ifenprodil enhances proton sensitivity of GluN1/GluN2B receptors (Pahk and Williams, 1997; Whitemore et al., 1997; Mott et al., 1998; Zheng et al., 2001). However, recent crystal structure analysis of the ifenprodil-bound GluN1/GluN2B ATD dimer revealed that ifenprodil binds at the interface of the GluN1/GluN2B ATD dimer, unlike zinc, which binds in a hydrophilic cleft formed by the bilobed GluN2B ATD (Karakas et al., 2009, 2011). Moreover, ifenprodil was found to be buried within the interface, suggesting that binding and unbinding of ifenprodil requires conformational change in the ATD dimer interface, supporting an induced-fit mechanism of ifenprodil binding (Karakas et al., 2011; Burger et al., 2012). A previous mutational study supports the finding
that GluN1 ATD partly forms the binding site for ifenprodil (Masuko et al., 1999). The gating mechanism of ifenprodil-induced conformational strain transduction from the ATD to the channel closure still remains to be elucidated.

We recorded single-channel currents in cell-attached, as well as outside-out, patches. At pH 7.9, we found that ifenprodil led to an overall reduction in the open probability. Additionally, GluN1/GluN2B receptors were found to exhibit negative correlations between the open and shut intervals, similar to that described previously for GluN1/GluN2A receptors (Schorge et al., 2005; Wyllie et al., 2006; Erreger and Traynelis, 2008). Moreover, the single-channel and macroscopic data fitted to a cyclic scheme with two uncoupled open states (hereafter “cyclic uncoupled” model), which incorporates the feature of conditional distribution and predicted that ifenprodil increases receptor desensitization and affects specific gating steps. We also found that in outside-out patches, ifenprodil reduced channel mean open time at pH 7.4 but not at pH 7.9, thus uncovering a novel hypothesis for the pH dependence of ifenprodil inhibition, whereby ifenprodil engages different gating mechanisms under alkaline versus acidic conditions.

**Materials and Methods**

**Expression of Recombinant NMDA Receptors**

Human embryonic kidney (HEK) 293 cells were maintained as previously described (Banke and Traynelis, 2003; Dravid et al., 2008). The cells were transiently transfected with Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY). Rat GluN1-1a (GenBank U11418, U08261), pCIneo vector; hereafter GluN1, provided by Dr. Stephen Heinemann, Salk Institute, San Diego, CA), GluN2B (Genbank U11419, Q00960; pcDNA3.1 vector, provided by Dr. Peter Seeburg, Max Planck Institute for Medical Research, Heidelberg, Germany), and green fluorescent protein in the ratio of 1:2:0.5 were used as previously described (Banke et al., 2005). Electrophysiology experiments were performed 24–48 hours after transfection.

**Electrophysiology**

Single-channel recordings from cell-attached, outside-out patches and whole-cell recordings were obtained from transfected HEK 293 cells at room temperature (22°C–25°C). An external solution containing (in millimolars) 150 NaCl, 3 KCl, 10 HEPES, and 0.5 CaCl₂ was used for the recordings. All the cell-attached recordings and whole-cell recordings were obtained at pH 7.9, whereas outside-out recordings were obtained at pH 7.4 and pH 7.9. The pH was adjusted with NaOH. This solution was supplemented with 0.01 mM EDTA to chelate trace amounts of zinc. The same external solution was used for outside-out recordings. This solution was supplemented with 0.01 mM EDTA to chelate trace amounts of zinc. The same external solution was used for outside-out recordings.

**Data Processing and Kinetic Modeling**

**Cell-Attached Recordings**

Recordings containing a single active channel were idealized using QUB software (www.qub.buffalo.edu). The presence of only a single active channel in the patches was further confirmed as previously described (Colquhoun and Hawkes, 1990; Dravid et al., 2008). Several cell-attached patches contained only a small number of longest-lived shut times. The single channel data were evaluated through correlations between successive open intervals by the runs test (Colquhoun and Sakmann, 1985) using Channel Lab (www synaptosoft.com). Open periods were separated into brief and long openings using a critical shut time (tcrit) of 0.5 ms, determined as previously described (Jackson et al., 1983). Openings shorter than 0.5 ms were given a value of 0, and open periods longer than 0.5 ms were assigned a value of 1. A run was defined as a series consisting entirely of one or more brief or long openings. Total 0 counts were designated as n₀, and total 1 counts were designated as n₁, thus the total number of runs (n) = n₀ + n₁. If the openings occur at random, the mean and variance of the number of runs (T) will be calculated as follows: Mean (T) = (2n₁n₀)/n₀ + 1; Var (T) = (n₁(n₀ − n₁))/n₀(n₀ + 1). For the test statistics, Z has a zero mean and unit standard deviation if the open duration lengths occur at random and can be defined as Z = (T – Mean (T))/Var(T)1/2. The Z value will be more than 2 if not random. We used a tₚₐₒ of 1193 ms to chop the idealized files to obtain a homogenous data set for performing maximum interval likelihood fitting (MIL; Qin et al., 1996). This tcrit was derived from the analysis of the shut time histogram from the longest control patch (35 minutes). The tcrit values of this patch were as follows: 0.3, 1.76, 64, and 1193 ms. The tcrit used for segmenting the data was also close to the tcrit of 1026 ms obtained using the time constants and areas from the global shut time histogram (Fig. 2) by a method that minimizes the misclassified events (Jackson et al., 1983). We also used a tcrit of 1193 ms for ifenprodil patches due to the relatively low number of >1000 ms events for individual patches in ifenprodil. A similar method of segmentation for both control and treated single-channel data has been previously used for GluN1/GluN2A receptors to study the effect of partial agonist and zinc (Erreger et al., 2005; Erreger and Traynelis, 2008). 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 Channel Lab, with an imposed dead time of 100 μs.

In single-channel recordings in the cell-attached mode, we observed that a small set of patches displayed higher overall open probability (overall open probability >0.2, n = 2 for each control and ifenprodil). This may be due to a dominant high mode in these patches, as previously reported (Amico-Ruvio and Poppescu, 2010). It would not be practical to evaluate the action of ifenprodil amid this...
variability, as it may obscure the results. Thus, we included only homogeneous patches with an overall probability of opening <0.2 (seven out of nine patches in the control and six out of eight in ifenprodil) for comparison of mean open time, open probability, and mean shut time, and for MIL fitting.

**Outside-Out Recordings.** We used recordings with single and multiple channels for analysis of channel properties, owing to the lack of long paired recordings with a single active channel. Of the six paired patches at pH 7.9 used in our analysis, two had one active channel and the remainder had two channels. Outside-out recordings containing two active receptors were subdivided using a $t_{crit}$ of 100 ms to obtain activation bursts with one active receptor, as previously described for GluN1/GluN2B 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. Recordings were also obtained at pH 7.4, which included patches containing two detectable channels. Due to the low number of events, these patches were used only for the analysis of mean open time. These recordings were subdivided using a $t_{crit}$ of 100 ms and analyzed as described previously.

**Analysis of Macroscopic Response Time Course**

Peak and steady-state responses and activation, deactivation, and desensitization time course were analyzed using Clampfit (pCLAMP 10.2). Macroscopic response wave forms were normalized, aligned, and averaged among recordings. The average control and ifenprodil wave form was then normalized with the steady-state currents to be equal to the open probability determined in one-channel cell-attached patches. Aggregated Markov models were fitted to individual wave forms using a nonlinear least sum of squares–fitting algorithm (Channel Lab). Fitting was performed using a Runga-Kutta numerical integrator to simulate each response wave form at each iteration using the same set of rate constants. Binding and unbinding rates and desensitization rates were set as free parameters, and all other rates were fixed to those obtained from MIL fits to the single-channel data in a manner similar to that previously described for NMDA receptors (Dravid et al., 2008).

**Statistical Analysis**

All values are expressed as the mean ± S.E.M. Data were compared using an unpaired t test for the cell-attached patches and a paired t test for outside-out data and macroscopic current profiles. Values of $P < 0.05$ were considered significantly different.

**Results**

**Ifenprodil Reduces the Open Probability of GluN1/GluN2B Receptors in Cell-Attached Recordings.** All cell-attached patches used for analysis were one-channel patches obtained at pH 7.9. To determine the effect of ifenprodil on GluN1/GluN2B gating, we first analyzed the apparent mean open time, apparent mean shut time, and the probability of opening from cell-attached recordings with only one active channel in the absence ($n = 7$) and presence ($n = 6$) of 3 μM ifenprodil (Fig. 1). The mean open time (±S.E.M.) in control patches was found to be 0.92 ± 0.14 ms (81,412 events). In the presence of ifenprodil, there was no significant change in the mean open time (0.98 ± 0.25 ms; 17,370 events). The mean shut time was found to significantly increase from 24.2 ± 4.8 ms in control patches (81,415 events) to 70.2 ± 16.5 ms in ifenprodil patches (17,376 events, $P < 0.05$, unpaired t test). The open probability, measured over the entire length of recordings, was found to be reduced from 0.045 ± 0.01 in control patches to 0.019 ± 0.006 in ifenprodil patches ($P < 0.05$, unpaired t test). Thus, ifenprodil led to a 58% reduction in open probability, similar to that previously described (Legendre and Westbrook, 1991). The amplitude of openings was unaffected by ifenprodil, with amplitude being 7.0 ± 0.5 pA for control patches ($n = 9$) and 6.0 ± 0.5 pA for ifenprodil ($n = 8$) (data not shown). Thus, ifenprodil inhibition of GluN1/GluN2B receptors appears to be solely due to the reduction in the probability of opening of the receptor at pH 7.9.

It should be noted that the open probability and mean open time in our patches is closer to the “low” mode of GluN1/GluN2B reported previously (Amico-Ruvio and Popescu, 2010). One possible explanation for this effect could be the difference in Ca$^{2+}$ concentration used by Amico-Ruvio and Popescu (2010) and that used in our experiments. They used 0 Ca$^{2+}$ in their study, whereas our cell-attached recordings were performed in an extracellular solution containing 0.5 mM Ca$^{2+}$. It is possible that the extracellular Ca$^{2+}$ concentration influences modal gating and reduces the overall activity of NMDA receptors, which is in agreement with previous studies demonstrating that extracellular Ca$^{2+}$ reduces channel conductance and activity (Premkumar and Auerbach, 1996; Wyllie et al., 1996).

**Ifenprodil Increases the Occupancy of Long-Lived Shut States in Cell-Attached Recordings.** Assessing the characteristics of the open and shut times can provide important information to assess gating mechanisms and the effect of modulators. The open and shut time histograms were fitted using a maximum likelihood method (Colquhoun and Sigworth, 1995) (Fig. 2). The global open time histogram was best fitted by the sum of three exponential components, and the composite shut time histogram was fitted by five exponential functions (Fig. 2). The global shut time fit clearly indicates a shift in the area of $r_2$ by ifenprodil, which can be observed in Fig. 2. Individual shut time histograms were subsequently fitted to 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 thus were fitted with only four components. Therefore, to maintain consistency in averaging fitted time constants across different patches, we used a $t_{crit}$ of 1193 ms (see Materials and Methods) to subdivide the data and fitted all the shut time events shorter than 1193 ms to four exponential functions. In addition, the open time histogram for individual patches could be fitted to two exponential components, as opposed to three exponential components for the global histogram. This suggests that components in individual patches differed such that when the patches were pooled, it led to the appearance of three exponential components for the global histogram. This suggests that extracellular Ca$^{2+}$ and not the time constants themselves is distinct from the effect of zinc at GluN1/GluN2A receptors (Erreger and Traynelis, 2008).

**Effects of Ifenprodil in Excised Outside-Out Recordings (pH 7.9 and 7.4).** Although recordings in the cell-attached mode have the advantage of keeping the intracellular milieu and receptor-scaffold interactions intact, paired recordings under outside-out conditions may provide an opportunity to design a paired comparison between control and ifenprodil
conditions on the same patch. Therefore, we tested whether the effects of ifenprodil on GluN1/GluN2B kinetics observed in the cell-attached recordings were conserved in the outside-out recording condition at pH 7.9 (Fig. 3). We used recordings of both one and two channels for analysis of channel properties in outside-out patches (see Materials and Methods). Global histograms were obtained by pooling all events from patches containing one active receptor and events measured from data subdivided using a tcrit of 100 ms from patches containing two active receptors. The global open time histogram was best fitted by a sum of three exponential components, and the composite shut time histogram was fitted by a sum of five exponential functions (Fig. 3). Similar to cell-attached recordings, the global shut time fit depicts a reduction in the area of $t^2$. No statistically significant reduction in the mean open time or channel open probability was observed in the presence of ifenprodil (see Materials and Methods). Global histograms were obtained by pooling all events from patches containing one active receptor and events measured from data subdivided using a tcrit of 100 ms from patches containing two active receptors. The global open time histogram was best fitted by a sum of three exponential components, and the composite shut time histogram was fitted by a sum of five exponential functions (Fig. 3). Similar to cell-attached recordings, the global shut time fit depicts a reduction in the area of $t^2$. No statistically significant reduction in the mean open time or channel open probability was observed in the presence of ifenprodil (Fig. 4A). 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 higher in ifenprodil ($P < 0.05$; Fig. 4A). Thus, the negative modulation of GluN1/GluN2B receptors by ifenprodil at pH 7.9 is due to the increase in the shut durations of the receptor, in agreement with our cell-attached results showing that ifenprodil increases the occupancy of the receptor in the long-lived shut conformations. Previous studies, however, have reported that ifenprodil reduces the mean open time of NMDA receptors (Legendre and Westbrook, 1991; Pina-Crespo and Gibb, 2002). A main difference in these previous studies is that recordings were performed at pH 7.4, as compared with pH 7.9, which is what we have used. Thus, we recorded at pH 7.4 to test whether there could be a pH-dependent effect of ifenprodil on mean open time. Interestingly, at pH 7.4, we found a significant reduction in mean open time by ifenprodil ($P < 0.05$; Fig. 4B). Thus, ifenprodil reduces the stability of the open state of the receptor depending on the degree of protonation.

We also observed that the mean open time and open probability were higher for excised patches compared with cell-attached patches, suggesting that in the 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 distributions 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 differences in recording configurations may affect the stability of the open state, but may not influence the gating steps leading to channel pore dilation.

**GluN1/GluN2B Receptors Display Conditional Distribution in Cell-Attached Recording.** In the 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; Beato et al., 2002, 2004; Hatton et al., 2003; Burzomato et al., 2004). Similar negative correlations have previously been reported for rat hippocampal NMDA receptors (Gibb and Colquhoun, 1991) and recombinant GluN1/GluN2A NMDA receptors (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 between open times in GluN1/GluN2B receptors in cell-attached patches (see Materials and Methods). 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 a 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, the presence of a negative correlation is a feature common to GluN1/GluN2A and GluN1/GluN2B receptors. The presence of correlations in the data suggests that the two open states are most likely connected to two different shut states. This information has an important impact on connectivity of states during kinetic modeling.

To further ascertain the existence of correlations in the data, we constructed conditional distributions of adjacent intervals (Fig. 5A). The shut time fitting of the longest 35-minute control patch was used to determine the \( t_{\text{crit}} \) values, as previously described in Materials and Methods (Jackson et al., 1983; Gibb and Colquhoun, 1991). 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 1193–10,000 ms as the shut time ranges to examine the conditional distribution. The average ± S.E.M. of apparent mean open time for all the patches in a given shut time range is presented in Fig. 5A. 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 times adjacent to a brief shut time range (0.3–1.76 ms, dark histogram) and adjacent to a longer

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**TABLE 1**

<table>
<thead>
<tr>
<th>Time Constants (ms) and Areas (%)</th>
<th>Control</th>
<th>Ifenprodil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shut time</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.14 ± 0.04</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>1.1 ± 0.08</td>
<td>3.71 ± 1.5</td>
</tr>
<tr>
<td>3</td>
<td>25 ± 3.7</td>
<td>37 ± 6.0</td>
</tr>
<tr>
<td>4</td>
<td>261 ± 29</td>
<td>257 ± 39</td>
</tr>
<tr>
<td>a1</td>
<td>8.1 ± 1.8</td>
<td>15 ± 4.1</td>
</tr>
<tr>
<td>a2</td>
<td>49 ± 2.6</td>
<td>20 ± 2.9***</td>
</tr>
<tr>
<td>a3</td>
<td>37 ± 1.8</td>
<td>47 ± 3.7*</td>
</tr>
<tr>
<td>a4</td>
<td>5.2 ± 1.1</td>
<td>18 ± 4.7*</td>
</tr>
<tr>
<td><strong>Open time</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.96 ± 0.17</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>a1</td>
<td>14 ± 2.4</td>
<td>16 ± 3.3</td>
</tr>
<tr>
<td>a2</td>
<td>86 ± 2.4</td>
<td>84 ± 3.4</td>
</tr>
</tbody>
</table>

\* \( p < 0.05 \); \*** \( p < 0.001 \).

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**Fig. 2.** Ifenprodil increases the occupancy of GluN1/GluN2B receptors in the long-lived shut states. The single-channel currents from cell-attached patches containing one active GluN1/GluN2B receptor were idealized to generate dwell time histograms. A dead time of 100 µs was imposed on the idealized data files. The open time histogram was fitted by a sum of three exponential components: control: \( n = 7,81,412 \) open events; ifenprodil: \( n = 6,17,370 \) events. The composite shut time histogram was fitted by a sum of five exponential functions: control: \( n = 7,81,415 \) closed periods; ifenprodil: \( n = 6,17,376 \) closed periods. The time constants and percent area are shown in the inset. After applying a \( t_{\text{crit}} \) of 1193 ms, the dwell times from each patch were individually fitted. Ifenprodil was found to significantly reduce the area of \( \tau_{2} \) and increase the area of \( \tau_{3} \) and \( \tau_{4} \) (Table 1).
shut time range (1.76–64 ms, gray 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 (Fig. 3). However, the area of the shorter time constant was greater for the longer shut time range, suggesting that the apparent mean open time was dependent on the shut time range.

Conceptual Models of GluN1/GluN2B Receptor Activation. In recent years, a number of conceptual models for NMDA receptor gating have been proposed (Banke and Traynelis, 2003; Popescu and Auerbach, 2003; Auerbach and Zhou, 2005; Erreger et al., 2005; Schorge et al., 2005; Dravid et al., 2008; Kussius and Popescu, 2009). Together with structural information, these conceptual models may allow determination of steps involved in receptor activation that define macroscopic currents and synaptic activity. Moreover, kinetic analysis may provide interpretation of potential actions of gating modulators. A linear model of NMDA receptor gating has been proposed (Popescu and Auerbach, 2003; Erreger et al., 2005), which may signify the simultaneous concerted conformational changes in the entire receptor during the activation pathway (Kussius and Popescu, 2009) (scheme 1; Fig. 5C). However, one of the first cyclic models proposed by Banke and Traynelis (2003) suggested that the receptor underwent two independent conformational changes, presumably corresponding to GluN1 and GluN2 subunits, after

Fig. 3. Effects of ifenprodil on dwell time histograms in the excised outside-out recordings. (A) Representative steady-state single-channel recording from HEK 293 cells expressing GluN1/GluN2B receptors in outside-out mode obtained with glutamate and glycine [1 mM and 100 μM, respectively; holding potential = −70 mV, filtered at 8 kHz (2 kHz for presentation), digitized at 40 kHz, duration 5 s] under control conditions. (B) Paired recording after application of ifenprodil (3 μM). Openings are downward. (C) Global open and shut time histograms. The global open time histogram was fitted by a sum of three exponential functions: control: n = 6, 46,788 open events; ifenprodil: n = 6, 23,802 events. The composite shut time histogram was fitted by a sum of five exponential components: control: 46,796 closed intervals; ifenprodil: 23,743 intervals. A dead time of 100 μs was imposed on the idealized data files.

Fig. 4. Effects of ifenprodil (IFN) on channel kinetics in the excised outside-out recordings. (A) Effects of ifenprodil on the GluN1/GluN2B receptor activity at pH 7.9. Graphs depicting the mean open time, open probability, and mean shut times of six paired control and ifenprodil patches (see Materials and Methods). Only the mean shut time was found to be significantly different (paired t test, P < 0.05). (B) At pH 7.4, ifenprodil significantly reduced the mean open time of GluN1/GluN2B receptors (n = 10; paired t test, P < 0.05).
which the channel pore opens. Scheme 2 presented in Fig. 5C is based on the same idea, but includes an additional shut state in which both conformational changes have occurred, which further leads to the sequential open states. An extension of this model was suggested by Schorge et al. (2005), who took the observed correlations in GluN1/GluN2A receptors into consideration and connected two open states separately from two gateway states (scheme 3). All the models tested contain two desensitized states to account for the long-lived shut states that we observed in our patches (Fig. 5C), similar to those previously used for GluN1/GluN2C, GluN1/GluN2A, and GluN1/GluN2B receptors (Dravid et al., 2008, 2010; Kussius and Popescu, 2009; Amico-Ruvio and Popescu, 2010).

We fitted the three previously proposed gating models for NMDA receptors (schemes 1, 2, and 3; Fig. 5C) to the cell-attached single-channel data to evaluate the effects of ifenprodil on GluN1/GluN2B 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 three schemes, the data have been fitted to describe the same number of rate constants, since microscopic reversibility was imposed for the loops in schemes 2 and 3. It should be noted that the two open states in the “cyclic uncoupled” model arise from two different shut states and have different stabilities, with open state O1 shorter in duration compared with open state O2. The opening rate for the long-lived O2 state was found to be between 25,000 and 35,000 s⁻¹ for the control and ifenprodil patches (Fig. 6), which is similar to the rate previously reported by Schorge et al., 2005. The two open states most likely represent different receptor conformations but might...
have the same channel pore size, since only one conductance level was observed in the recording.

As a first step to investigate the effect of ifenprodil on GluN1/GluN2B gating, we addressed the quality of fit to the three models (schemes 1, 2, and 3) by performing MIL fitting to the idealized data from the longest (35-minute) patch. As seen in Fig. 5C, the log likelihood values (LL) were 305,611.5; 305,611; and 306,350 for schemes 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 relatively better quality fit compared with the other two models. Additionally, MIL fits to schemes 1 and 2 yielded similar rate constants as observed previously (Dravid et al., 2008, 2010); therefore, we have considered only two models, the linear model (scheme 1) and the cyclic uncoupled model (scheme 3), for further analysis. 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, to maintain consistency in MIL fits across different patches, we used a $t_{\text{crit}}$ of 1193 ms (see Materials and 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 (control = 9, ifenprodil = 8) that had been segmented using the $t_{\text{crit}}$, we found that both the linear and cyclic uncoupled models provided a good quality fit to the data, with cyclic uncoupling having a relatively higher LL compared with the linear model.

Effect of Ifenprodil on GluN1/GluN2B Receptor Gating. Next, we evaluated the effect of ifenprodil on receptor function in the framework of the linear and cyclic uncoupled models of GluN1/GluN2B receptor gating. As mentioned previously, a $t_{\text{crit}}$ of 1193 ms was applied to the patches for MIL fits, and only one desensitized state (D2) was included in the models to account for the loss of the long-lived shut state that accompanies the use of $t_{\text{crit}}$ (Figs. 6B, 6C). First, upon fitting the idealized data to a linear model, we found that only the forward rate of a fast gating step ($k_2^+$) was reduced ($P < 0.05$) and all other rate constants were unaffected (Fig. 6B). In contrast, when fitting MIL to a cyclic uncoupled model (Fig. 6C), we found that the forward ($k_1^+$) rate of a fast gating step was reduced 2-fold, whereas the reverse rate ($k_2^-$) of a slow gating step was increased 2-fold ($P < 0.05$). In addition, the rate of entry into the desensitized state ($d_2^+\to S$) was increased ($P < 0.05$), similar to that predicted previously from macroscopic currents (Kew et al., 1996). The channel closing rates were unaffected by ifenprodil, in agreement with the lack of effect of ifenprodil on the mean open time in our data. The mean ± S.E.M. rate constants obtained from MIL fitting of the idealized data are presented in Table 2. A representative patch with the fitted dwell time histogram is shown in Fig. 6A. We also fitted a group of patches that had a reasonable number of long-lived shut events, beyond the $t_{\text{crit}}$ of 1193 ms, to the entire model containing both desensitized states, and found the same rate constants changed in each of the schemes.

The rates derived from the MIL fit for the linear and cyclic uncoupled models 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 used patches that 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, Fig. 5, schemes 1 and 3). The simulations accurately predicted a reduction in the open probability of GluN1/GluN2B receptors in the presence of ifenprodil. Monte Carlo simulations using the cyclic uncoupled model predicted that ifenprodil would reduce the mean open time from 1.066 (control) to 1.014. The open probability was reduced from 0.03 (control) to 0.008 in the presence of ifenprodil. Simulations performed using the linear model predicted the mean open time to be 0.961 for control and 1.029 for ifenprodil, and the open probability to be 0.018 for control and 0.007 for ifenprodil. A dead time of 150 ms was imposed.

**Macroscopic Time Course and Effect of Ifenprodil.** We next evaluated the effect of ifenprodil on whole-cell ensemble currents from GluN1/GluN2B receptors (Fig. 7). Ifenprodil (3 μM) inhibited the peak whole-cell current by 68% ± 8% ($n = 5$). Ifenprodil significantly prolonged the deactivation time course of glutamate from 362 ± 42 ms to 590 ± 67 ms ($P < 0.01$; Fig. 7B), in agreement with previous finding concerning ifenprodil and the effect of ATD on deactivation rate (Kew et al., 1996; Yuan et al., 2009). Moreover, the ratio of steady-state current responses to peak current responses ($I_{\text{SS}}/I_{\text{peak}}$) was significantly lower for ifenprodil (0.62 ± 0.08) compared with control (0.75 ± 0.05, $P < 0.05$; Fig. 7B), in agreement with an increase in desensitization by ifenprodil (Kew et al., 1996; Zheng et al., 2001). No significant change in rise time was observed in the presence of ifenprodil (9.1 ± 1.8 ms) compared with control (8.8 ± 0.9 ms). We next tested whether the Markov models obtained by fitting of the single-channel data were able to describe macroscopic currents. Agonist binding and unbinding steps were included in schemes 1 and 3 for control and ifenprodil models (Fig. 7C). Both linear and cyclic uncoupled models were able to describe the macroscopic activation, deactivation, and steady-state open probability in control and ifenprodil conditions (Fig. 7C, Table 3). Both models also predicted a reduction in apparent $K_d$ (binding affinity) of glutamate, as previously described (Kew et al., 1996), which may be explained by slower deactivation in the presence of ifenprodil. However the cyclic uncoupled model had lower normalized residual sums of squares, indicating an overall better fit to the macroscopic data, and more accurately predicted the $I_{\text{SS}}/I_{\text{peak}}$ ratio in comparison with the linear model (Table 3).

**TABLE 2**

Hidden Markov maximum interval likelihood fitting of the steady-state currents

Idealized current records were fitted to the gating scheme as described in Fig. 5. All rates have units of s$^{-1}$. Data are the mean ± S.E.M. from seven control recordings and six ifenprodil recordings, each containing one active channel, fitted individually. The rates were compared by unpaired $t$ test.

<table>
<thead>
<tr>
<th>Rates (s$^{-1}$)</th>
<th>Scheme 1 Linear</th>
<th>Scheme 3 Cyclic Uncoupled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ifenprodil</td>
</tr>
<tr>
<td>$k_1^+$</td>
<td>90 ± 15</td>
<td>120 ± 30</td>
</tr>
<tr>
<td>$k_1$</td>
<td>495 ± 105</td>
<td>660 ± 295</td>
</tr>
<tr>
<td>$k_2^+$</td>
<td>1760 ± 440</td>
<td>630 ± 200*</td>
</tr>
<tr>
<td>$k_2$</td>
<td>4690 ± 1420</td>
<td>3240 ± 835</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>5650 ± 615</td>
<td>4625 ± 555</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>18,425 ± 2900</td>
<td>22,630 ± 1495</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>6185 ± 810</td>
<td>6125 ± 1150</td>
</tr>
<tr>
<td>$d_2^+$</td>
<td>2160 ± 450</td>
<td>1860 ± 470</td>
</tr>
<tr>
<td>$d_2$</td>
<td>33 ± 10</td>
<td>40 ± 20</td>
</tr>
<tr>
<td>$d_2^-$</td>
<td>4.1 ± 0.3</td>
<td>5.4 ± 0.6</td>
</tr>
</tbody>
</table>

* $P < 0.05$. 

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Ifenprodil on GluN1/GluN2B Gating 17

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There are several caveats for our modeling and hypothesis. First, we have not considered other possible models of NMDA receptor gating, such as a parallel scheme with ifenprodil bound or unbound state (for example, Banke et al., 2005; Erreger et al., 2005; Erreger and Traynelis, 2008). Additionally, we have assumed the receptor to be fully bound to ifenprodil, which may not be the case, since at 3 μM with the IC50 being 300 nM, the occupancy is likely to be around 90%. Second, we have ignored the potential role of protonation in gating. At pH 7.9, approximately 25% of the GluN1/GluN2B receptors are still inhibited (Jang et al., 2004). Not only do protons inhibit GluN1/GluN2B receptors, they also affect ifenprodil IC50 (Mott et al., 1998). Thus, the kinetic models we have tested are too simplified and should, therefore, be interpreted with caution. Third, it has been shown that the briefest open and shut times can be influenced by the method of idealization (Schorge et al., 2005). Thus, our rate of digitization and filtering and method of idealization may have led to loss of crucial information for kinetic modeling. Fourth, kinetic analysis was performed using only the cell-attached patches and not paired outside-out patches.

**TABLE 3**

Fitting of macroscopic GluN1/GluN2B current response with and without ifenprodil

The normalized residual sum of squares (indicated as "residual") were derived from the macroscopic fitting with the gating rates fixed to those derived from MIL fits (Table 2); the desensitization (d) and agonist binding (b+) and unbinding (b-) rates were set as free parameters. The agonist binding rates are in μM⁻¹s⁻¹ and all other rate constants are s⁻¹. The Kd is in μM and calculated as b⁻/b++. The desensitization rate was constrained to the following range: d1⁺ and d1⁻ = 0.1–10, d2⁺ = 5–250, and d2⁻ = 1–25.

<table>
<thead>
<tr>
<th>Rates</th>
<th>Experiment</th>
<th>Scheme 1 Linear</th>
<th>Scheme 3 Cyclic Uncoupled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ifenprodil</td>
<td>Control</td>
</tr>
<tr>
<td>b⁺</td>
<td>1.5</td>
<td>3.1</td>
<td>2.1</td>
</tr>
<tr>
<td>b⁻</td>
<td>2.5</td>
<td>1.2</td>
<td>4.4</td>
</tr>
<tr>
<td>d₁⁺</td>
<td>0.16</td>
<td>0.11</td>
<td>0.55</td>
</tr>
<tr>
<td>d₁⁻</td>
<td>0.45</td>
<td>0.37</td>
<td>0.93</td>
</tr>
<tr>
<td>d₂⁺</td>
<td>6.9</td>
<td>6.0</td>
<td>88</td>
</tr>
<tr>
<td>d₂⁻</td>
<td>21</td>
<td>7.7</td>
<td>19</td>
</tr>
<tr>
<td>Residual</td>
<td>2.64 × 10⁻⁶</td>
<td>3.02 × 10⁻⁶</td>
<td>2.16 × 10⁻⁶</td>
</tr>
<tr>
<td>Kd</td>
<td>1.7</td>
<td>0.38</td>
<td>2.1</td>
</tr>
<tr>
<td>P₀ (SS)</td>
<td>0.045</td>
<td>0.019</td>
<td>0.047</td>
</tr>
<tr>
<td>MOT</td>
<td>0.92</td>
<td>0.98</td>
<td>0.82</td>
</tr>
<tr>
<td>Iₜₛₛ/Iₚₑᵃᵏ</td>
<td>0.75</td>
<td>0.62</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Iₚₑᵃᵏ peak current; Iₜₛₛ steady-state current; MOT, mean open time; P₀, open probability.
which may have provided more controlled information. This limitation arose from the lack of a sufficient number of outside-out patches with one active channel to perform MIL fits. Fifth, we have used only one desensitized state in the models for conducting MIL fits to account for the low number of events in the longest shut state, which may affect the rate constants. Despite these limitations, the use of cell-attached patches, as well as outside-out patches, strengthens our results and interpretation.

Discussion

In the current study, we addressed the effect of ifenprodil on GluN1/GluN2B receptor gating. There are four significant findings from our investigation. First, the inhibition of GluN1/GluN2B receptors by ifenprodil at pH 7.9 can be attributed solely to an increased occupancy of long-lived shut time. Ifenprodil leads to reduction in the open probability of the receptor, with no significant influence on mean open time and amplitude, as observed in the cell-attached recordings. Interestingly, there was no shift in the time constants of the shut time histogram. In contrast, ifenprodil significantly changed the area of the exponential time constants. Second, ifenprodil modified the mean open time in outside-out patches in a pH-dependent manner, producing a significant reduction in mean open time at pH 7.4 but not at pH 7.9. This raises the possibility that this unique effect of ifenprodil on GluN1/GluN2B gating may underlie the pH-dependent inhibition of GluN1/GluN2B macroscopic currents by ifenprodil. Third, similar to GluN1/GluN2A receptors (Schorge et al., 2005), negative correlations between the open and shut intervals are observed in GluN1/GluN2B receptors, both in control and ifenprodil patches, suggesting that the two open states arise from two different closed states. Fourth, a cyclic uncoupled model was able to fit both single-channel and macroscopic GluN1/GluN2B receptor activity both in the absence and presence of ifenprodil, which was in agreement with correlations and conditional distribution found in our data and predicted that ifenprodil increased receptor desensitization and had unique effects on gating steps.

Allosteric Modulation by Ifenprodil. Ifenprodil was identified as a GluN1/GluN2B selective inhibitor (Williams, 1993) and originally was thought to solely bind to the GluN2B ATD (Perin-Dureau et al., 2002; Malherbe et al., 2003; Wong et al., 2005; Ng et al., 2008). Ifenprodil preferentially binds the receptor in open and desensitized conformations (Kew et al., 1996) and reduces the duration of the long openings and open probability of the receptor in cultured hippocampal neurons (Legendre and Westbrook, 1991) and in hippocampal slices from newborn rats (Pina-Crespo and Gibb, 2002). However, we did not find a significant decrease in mean open time by ifenprodil, which may be explained by a difference in the pH of our recording solution (pH 7.9). At physiologic pH, about 50% of the GluN2B-containing NMDA receptors are inhibited (Traynelis et al., 1995; Mony et al., 2009), and both zinc and ifenprodil increase sensitivity to proton block (Park and Williams, 1997; Mott et al., 1998; Choi and Lipton, 1999; Low et al., 2000; Zheng et al., 2001; Erreger and Traynelis, 2008). Thus, a difference in the pH of the recording condition may affect the influence of ifenprodil on mean open time. Indeed, we found that at pH 7.4, ifenprodil significantly reduced mean open time in outside-out patches.

Together with previous findings, it can be concluded that protons and ATD-binding inhibitors like zinc and ifenprodil may have a unique effect on GluN1/GluN2A and GluN1/GluN2B receptor gating. First, protons have been reported to affect only the open probability of the GluN1/GluN2B receptors, with no significant reduction in the mean open time (Banke et al., 2005), similar to our findings with ifenprodil at pH 7.9. In contrast, allosteric inhibition of GluN1/GluN2A receptors by protons and zinc involves a reduction in mean open time (Erreger and Traynelis, 2008; Amico-Ruvio et al., 2011). Second, zinc at the GluN1/GluN2A receptors has been found to modify the shut time constants, as well as their areas (Erreger and Traynelis, 2008; Amico-Ruvio et al., 2011). In contrast, we found that ifenprodil did not modify the time constants, only the area of the shut time constants, specifically those represented by $\gamma_3$ and $\gamma_4$. It should be noted that recent crystal structure data demonstrated that ifenprodil is buried deep in the ATD dimer interface within a hydrophobic region (Karakas et al., 2011), leading to the proposal of an induced-fit mechanism of binding. In contrast, zinc binds to the bilobed cleft of GluN2A ATD; it has been proposed that binding to the cleft leads to closure of the clampshell-like structure (Karakas et al., 2009). Based on the lack of effect on time constants by ifenprodil at pH 7.9, we propose that binding of ifenprodil to the ATD dimer interface restricts the transition of the receptor to an open state and increases the dwell time of the receptor in intrinsically occurring closed conformations, rather than inducing a novel receptor conformation. On the other hand, zinc binding to the ATD clampshell may lead to closure of the clampshell and generation of a novel receptor conformation that occurs infrequently during typical receptor gating.

GluN1/GluN2B Receptor Gating and Influence of Ifenprodil. We found that the duration of a sojourn in an open state was negatively dependent on the adjacent shut interval for the GluN1/GluN2B receptor in the cell-attached patches, which was maintained in the presence of ifenprodil. Negative correlations have been previously described for GluN1/GluN2A receptors (Gibb and Colquhoun, 1991; Schorge et al., 2005; Wyllie et al., 2006; Erreger and Traynelis, 2008) and have been accounted for by the gating model proposed by Schorge et al. (2005). Thus, the presence of a negative correlation identifies another common gating feature among GluN1/GluN2A and GluN1/GluN2B receptors. Interestingly, the cyclic uncoupled model (scheme 3) that accommodates the feature of negative correlations fitted the single-channel data with a higher LL (Fig. 5) and the macroscopic data with a lower residual sums of squares (Table 3). The cyclic uncoupled model indicated that ifenprodil reduces the forward rate of entry of the receptor from the initial agonist-bound closed state $C_1$ to $C_5$, which was a fast gating step (Fig. 6C). Although ifenprodil did not modify the slow forward $C_1$ to $C_2$ gating step, it increased the reverse $C_2$ to $C_1$ rate. Additionally, ifenprodil increased the rate of entry of the receptor into the desensitized state, in agreement with the macroscopic data (Fig. 7; Kew et al., 1996).

Upon agonist binding, the NMDA receptor undergoes a series of kinetically distinct conformational changes that precede pore opening. Banke and Traynelis (2003) originally proposed that the individual subunits undergo independent conformational changes. The faster shut rate constants were attributed to the GluN1 subunit, and slow ones to the GluN2
subunit. Indeed, it has been demonstrated structurally that the GluN1 and GluN2C subunits are positioned differently relative to the central and vertical axes of the channel pore (Sobolevsky et al., 2007; Salussolia et al., 2011; but see Chang and Kuo, 2008). Moreover, the crystal structure of the GluA2 receptor and its comparison with NMDA receptors suggests that GluN1 subunits are proximal whereas GluN2 subunits are distal, which may lead to a different degree of contribution in receptor gating (Sobolevsky et al., 2009; Riou et al., 2012). Thus, interpreting the cyclic uncoupled model in terms of this initial hypothesis would suggest that both GluN1 and GluN2 gating steps are modified by ifenprodil. This interpretation is also in agreement with the identification of ifenprodil binding to the GluN1/GluN2B ATD dimer interface (Masuko et al., 1999; Karakas et al., 2011; Burger et al., 2012). An alternative interpretation based on the linear model representing concerted movement of the entire receptor, involving the ligand binding domain closure and transmembrane movement followed by fast oscillations in the selectivity pore (Kussius and Popescu, 2009), is that ifenprodil selectively slows the movement of the transmembrane domains of the receptor, since only the forward rate ($k_2^+$) was significantly reduced. Interestingly, mutations in the transmembrane domain have been shown to affect proton sensitivity, an effect that is proposed to be downstream of ifenprodil binding (Low et al., 2003).

A report published while the current study was under review studied the effect of a saturating concentration of ifenprodil on GluN1/GluN2B gating and found some contrasting results compared with our study (Amico-Ruvio et al., 2012). First, there were differences in the mean open time and open probability under control conditions, with both being higher in comparison with our results; ifenprodil reduced both parameters. Additionally, the previous study did not observe an increase in macroscopic desensitization by ifenprodil. The overall shape of the single-channel shut time histograms were, however, quite comparable between the two studies. In addition to the use of saturating ifenprodil compared with the saturating concentration in our case, studies by Amico-Ruvio et al. (2012) were performed in the absence of extracellular Ca$^{2+}$, whereas we use a nominal 0.5 mM Ca$^{2+}$ in our recordings. Extracellular Ca$^{2+}$ concentration can affect NMDA channel gating (Premkumar and Auferberg, 1996; Wylie et al., 1996). Together, these results raise an important question regarding the influence of extracellular Ca$^{2+}$ on the mechanisms of NMDA receptor gating modifiers and NMDA receptor gating in general.

**Conclusion**

Together with the proposed induced-fit mechanism of ifenprodil binding to the dimer interface, our data indicate that, once bound, ifenprodil restricts further transition of the receptor to an open state and increases dwell time in an intrinsically occurring long-lived closed state. Our results also provide evidence that the pH-dependent effect of ifenprodil on mean open time may contribute to its therapeutically relevant property as a context-dependent inhibitor.

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**Authorship Contributions**

**Participated in research design:** Bhatt, Prakash, Dravid.

**Conducted experiments:** Bhatt, Prakash, Suryavanshi, Dravid.

**Performed data analysis:** Bhatt, Prakash, Dravid.

**Wrote or contributed to the writing of the manuscript:** Bhatt, Dravid.

**References**


