

An NR2B Point Mutation Affecting Haloperidol and CP101,606 Sensitivity of Single Recombinant N-Methyl-D-Aspartate Receptors¹

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Accepted for publication April 7, 1998 This paper is available online at <http://www.jpvet.org>

ABSTRACT

Haloperidol and ifenprodil are N-methyl-D-aspartate (NMDA) receptor (NR) antagonists with preference for the NR1/NR2B subunit combination. Previous investigations utilizing ¹²⁵I-MK801 binding assays with recombinant receptors distinguished certain structural determinants on the NR2B subunit for these two drugs, with glutamate 201 being critical for haloperidol sensitivity and arginine 337 being important for ifenprodil block. Other studies, however, suggested that these two sites pharmacologically overlap. In an attempt to resolve these discrepancies, we have characterized the actions of haloperidol and CP101,606, an ifenprodil analog, on the single-channel properties of NR1/NR2B(E201R) receptors transiently ex-

pressed in Chinese hamster ovary cells, because receptors formed by NR1/NR2B(R337K) appear to be nonfunctional. Haloperidol (10 μM) inhibited wild-type NR1/NR2B channels by decreasing the frequency of channel opening, whereas CP101,606 (0.5 μM) antagonized NR1/NR2B channel activity by decreasing both the open dwell time and the frequency of channel opening. The inhibitory actions of both drugs were virtually absent in the mutant NR1/NR2B(E201R) receptors. These results suggest that glutamate 201 is critical for both haloperidol and CP101,606 inhibition, thus demonstrating common features in the action of these two antagonists.

The NMDA receptor is a ligand-gated ion channel involved in excitatory neurotransmission, synaptic plasticity and neuronal cell death. Although the precise subunit composition and stoichiometry of this receptor are still being debated (Bébé *et al.*, 1995; Ferrer-Montiel and Montal, 1996; Premkumar and Auerbach, 1997), it is generally agreed that receptors in mammalian cells are formed by the co-assembly of the NR1 subunit with at least one type of NR2 subunit (Wafford *et al.*, 1993; Boeckman and Aizenman, 1994; Chazot *et al.*, 1994; Sheng *et al.*, 1994; McIlhinney *et al.*, 1996; Luo *et al.*, 1997). The type of NR2 subunit present in a functional receptor confers unique pharmacological and biophysical properties upon NMDA receptors (Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992; Stern *et al.*, 1992; Williams, 1993; Williams *et al.*, 1994; Burnashev *et al.*, 1995; Kuner and Schoepfer, 1996; Brimecombe *et al.*, 1997). For example, there are a number of substances that are more effective antagonists of

the recombinant NR1/NR2B subunit combination when compared with NR1/NR2A receptors (Williams, 1993; Lynch *et al.*, 1995; Lynch and Gallagher, 1996; Avenet *et al.*, 1996).

An example of such a drug is ifenprodil (fig. 1), a noncompetitive NMDA receptor antagonist (Carter *et al.*, 1988) and neuroprotective agent (Gotti *et al.*, 1988). This agent decreases single-channel activity and reduces channel open dwell time in a voltage-independent manner in native hippocampal NMDA receptors (Legendre and Westbrook, 1991). Studies utilizing recombinant receptors have revealed that this drug is highly selective for NR1/NR2B-containing receptors, as measured in the frog oocyte system (Williams, 1993) and in transfected HEK 293 cells (Gallagher *et al.*, 1996). Because ifenprodil also possesses nanomolar affinity for other neurotransmitter receptors such as adrenergic and serotonergic receptors (Chenard *et al.*, 1991), several related analogs with increased specificity for the NMDA receptor were recently synthesized. We have previously investigated the effects of one such compound, CP101,606 (Chenard *et al.*, 1995; fig. 1), on recombinant NMDA receptors expressed in CHO-K1 cells (Boeckman and Aizenman, 1996; Brimecombe *et al.*, 1997). This drug protected cells expressing NR1/NR2B, but not NR1/NR2A, receptors from the cytotoxicity that ensues after functional NMDA receptor expression, in a dose-

Received for publication February 27, 1998.

¹ This work was supported in part by NIH grants NS29365 (E.A.), DA07130 (D.R.L.), NS01789 (D.R.L.) and 1F32-DA05675 (M.J.G.) and by the National Alliance for Research on Schizophrenia and Depression (E.A., D.R.L.).

ABBREVIATIONS: CHO, Chinese hamster ovary; NMDA, N-methyl-D-aspartate; CP101,606, (1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol; NR, NMDA receptor subunit

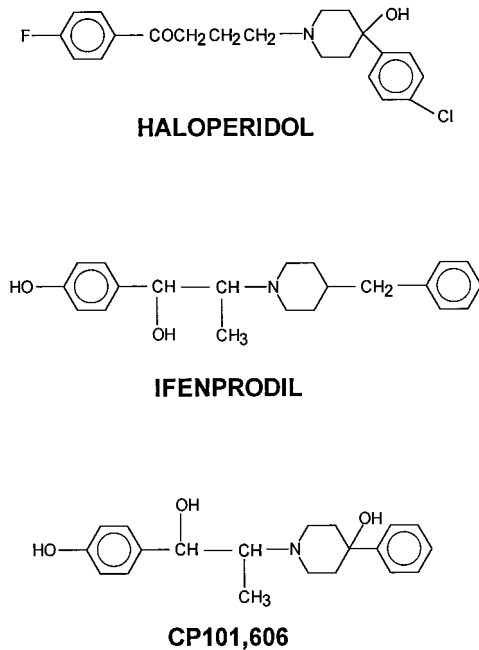


Fig. 1. Structures of haloperidol, ifenprodil and CP101,606.

dependent manner. Similar to the actions of ifenprodil on native receptors, CP101,606 inhibited NR1/NR2B channel activity by decreasing both the open dwell time of the channel and the frequency of channel opening. This drug did not drastically alter either NR1/NR2A or NR1/NR2C channel activity. Therefore, CP101,606 selectively inhibits NMDA receptors composed of NR1 and NR2B in a manner analogous to ifenprodil.

Haloperidol (fig. 1), a commonly used antipsychotic agent that antagonizes dopamine D_2 receptors, can also inhibit NMDA receptor activity in a subunit-selective manner, with NR2B-containing receptors displaying more sensitivity for the drug (Lynch and Gallagher, 1996; Ilyin *et al.*, 1996; Whittemore *et al.*, 1997). Haloperidol inhibits NMDA-induced native channel activity in rat cortical neurons by decreasing both the mean open dwell time and the frequency of channel opening in a voltage-independent manner (Ilyin *et al.*, 1996), similar to the actions of ifenprodil on recombinant and native NMDA receptors. Recent ^{125}I -MK801 binding assays using site-specific mutants have localized the putative sites of action of these two antagonists on the NR2B subunit, with arginine 337 being important for ifenprodil sensitivity and glutamate 201 being critical for haloperidol block (Gallagher *et al.*, 1996; Gallagher *et al.*, 1998). Other studies, however, have demonstrated that haloperidol inhibits [^3H]ifenprodil binding in adult rat brain membranes, suggesting that these two drugs may have pharmacologically overlapping binding sites (Coughenor and Cordon, 1997). In an effort to elucidate the pharmacological sites of action of these drugs, we have investigated whether mutations at arginine 337 and glutamate 201 in NR2B similarly affect the actions of haloperidol and CP101,606 at the single-channel level.

Materials and Methods

Tissue culture and transfection protocol. CHO-K1 cells (ATTC CCL61) were grown in Ham's F-12 nutrient medium with

10% fetal bovine serum and 1 mM glutamine (CHO medium) in 50- or 200-ml flasks. Cells were passaged at a 1:10 dilution at 80% confluence, approximately every 2 days, no more than 40 times. The cDNAs for the NMDA subunits NR1 and NR2B ($\epsilon 2$) were previously subcloned into mammalian expression vectors (Boeckman and Aizenman, 1994; Boeckman and Aizenman, 1996; Gallagher *et al.*, 1996; Gallagher *et al.*, 1997). The expression vector for a positive transfection marker protein, green fluorescent protein (GFP), was also generated previously (Brimecombe *et al.*, 1997). Recombinant NMDA receptors were transiently expressed in CHO cells by using LipofectAMINE (Gibco-BRL) reagent. Cells were seeded at 3×10^5 cells per well in six-well plates ~ 24 hr before transfection with 1.1 μg of total DNA and 5 μl of lipofectAMINE in 1 ml of serum-free CHO medium per 35-mm dish. The ratio of marker plasmid (pCI/GFP) to total DNA was 1:4.3, and the ratio of NR1 to NR2 subunits transfected was 1:3 (Cik *et al.*, 1993). After a 4- to 5-hr incubation at 37°C with the transfection solution, cells were refed with CHO medium containing 1 mM 5,7-dichlorokynurenic acid to prevent the cell death that accompanies NMDA receptor expression (Boeckman and Aizenman, 1996). Cells were used for recording ~ 40 to 50 hr after the start of the transfection.

Patch-clamp recordings. Electrophysiological measurements were performed at room temperature (25°C) with the outside-out configuration of the patch-clamp technique by utilizing 10- to 15-M Ω silicon-coated electrodes. Current signals were amplified by using an Axopatch 200 patch-clamp amplifier (Axon Instruments), filtered at 2 kHz with an 80-dB/decade low-pass Bessel filter, stored with a videotape system (Neuro Data), and later replayed and digitized at 10 kHz with a computer interface system (Digidata 1200, Axon Instruments). The reference electrode was a Ag-AgCl wire connected to the extracellular solution by a 2 M KCl/1% agarose bridge. The extracellular recording solution was nominally Mg^{++} -free and contained (in mM): NaCl, 150; KCl, 2.8; CaCl_2 , 1.0; HEPES, 10 and glycine 0.01 (pH adjusted to 7.2 with 0.3 N NaOH). The intracellular pipette solution contained (in mM): CsF, 140; ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10; CaCl_2 , 1.0 and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 (pH adjusted to 7.2 with CsOH). Drugs and chemical agents were

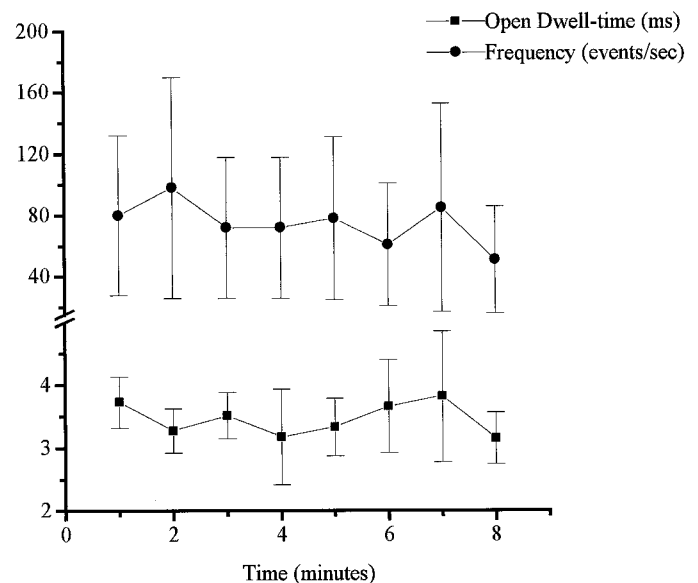


Fig. 2. Stability of two single-channel parameters over the normal time course of the experimental protocol in wild-type NR1/NR2B receptors. Patches excised from NR1/NR2B-transfected CHO cells were exposed to 10 μM NMDA continuously for 8 min. Recordings were analyzed at 1-min intervals for the duration of the exposure. Open dwell time and frequency of channel opening measurements were obtained for each interval. Data represent the means \pm S.E.M. of three separate patches.

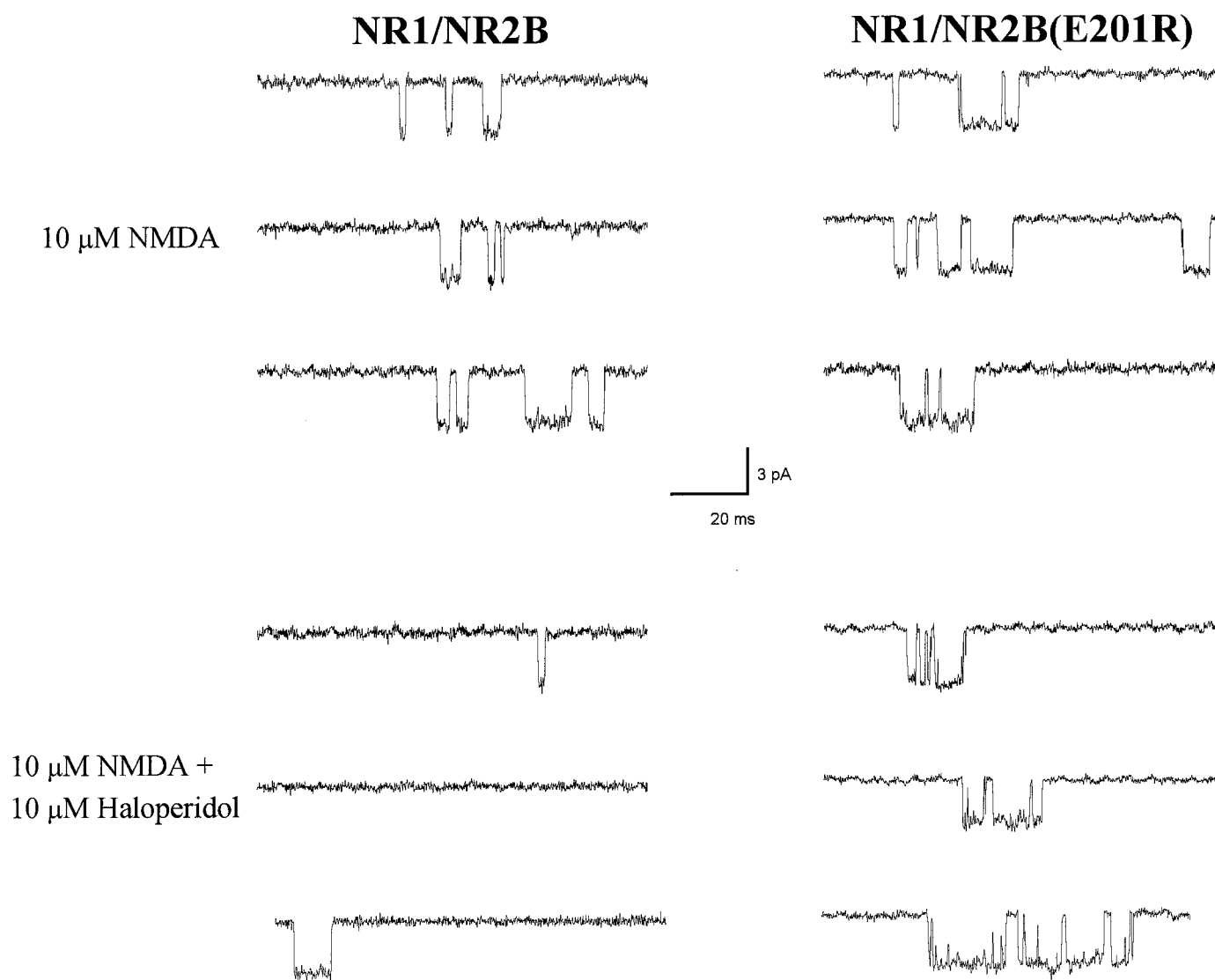


Fig. 3. Representative single-channel events elicited by NMDA in the absence or presence of haloperidol. Single-channel recordings were obtained from outside-out patches excised from cells transfected with either NR1/NR2B or NR1/NR2B(E201R). Events were elicited by $10 \mu\text{M}$ NMDA in the absence or presence of $10 \mu\text{M}$ haloperidol at -60 mV .

dissolved in the extracellular solution. NMDA ($10 \mu\text{M}$), haloperidol ($10 \mu\text{M}$) and (1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol (CP101,606; $0.5 \mu\text{M}$) were applied to the patch by complete bath exchange.

Data analysis. Single-channel analysis was performed by using pClamp6 software (Axon Instruments) that utilized a 50% threshold criterion. In total, data were gathered from 25 patches obtained from transfected CHO cells. Data from other patches lost before the completion of a treatment protocol were not utilized, because each patch served as its own control. Normally, 200 to 500 events (range of 30-sec to 2-min traces) were analyzed per single treatment, although many records contained a much larger number of events. Multiple openings, when present, constituted $<3\%$ of all openings for a given treatment. Amplitude histograms obtained from idealized traces were most commonly fit with a single Gaussian function. Most (72%) open dwell-time histograms were best fit with a single exponential function by using a simplex maximum-likelihood fitting routine on log-transformed binned data (six bins per decade). An F statistic obtained from a χ^2 analysis was utilized to determine the simplest fit of the data. When an open dwell-time histogram was better fit by the sum of two exponentials (28% of all histograms), the weighted mean

open time was utilized for the necessary calculations. In our previous work with recombinant NMDA receptors expressed in CHO cells, the vast majority of the open dwell-time histograms were best fit with a single exponential function (Brimecombe *et al.*, 1997) when NMDA was used as the agonist. Previous work on recombinant receptors expressed in oocytes or HEK 293 cells (Stern *et al.*, 1994) have reported multiple open dwell times when glutamate is used as the ligand, although the majority of events (70%) have an open dwell time that is very similar to our time constant. Events briefer than $180 \mu\text{sec}$ (twice the rise time of the filter) were ignored. We did not obtain recordings after removal of the antagonists used, because CP101,606 in particular is quite difficult to wash out completely. We therefore conducted a series of experiments aimed at determining the stability of our patches. Three patches excised from cells transfected with NR1/NR2B were continuously exposed to $10 \mu\text{M}$ NMDA alone. Recordings were obtained for 8 min (a time period actually longer than the normal experimental protocol performed in the present paper) in the continuous presence of agonist. Traces were analyzed in 1-min intervals, with the open dwell time and frequency of channel opening analyzed for each segment. As demonstrated in figure 2, both parameters were relatively stable throughout the

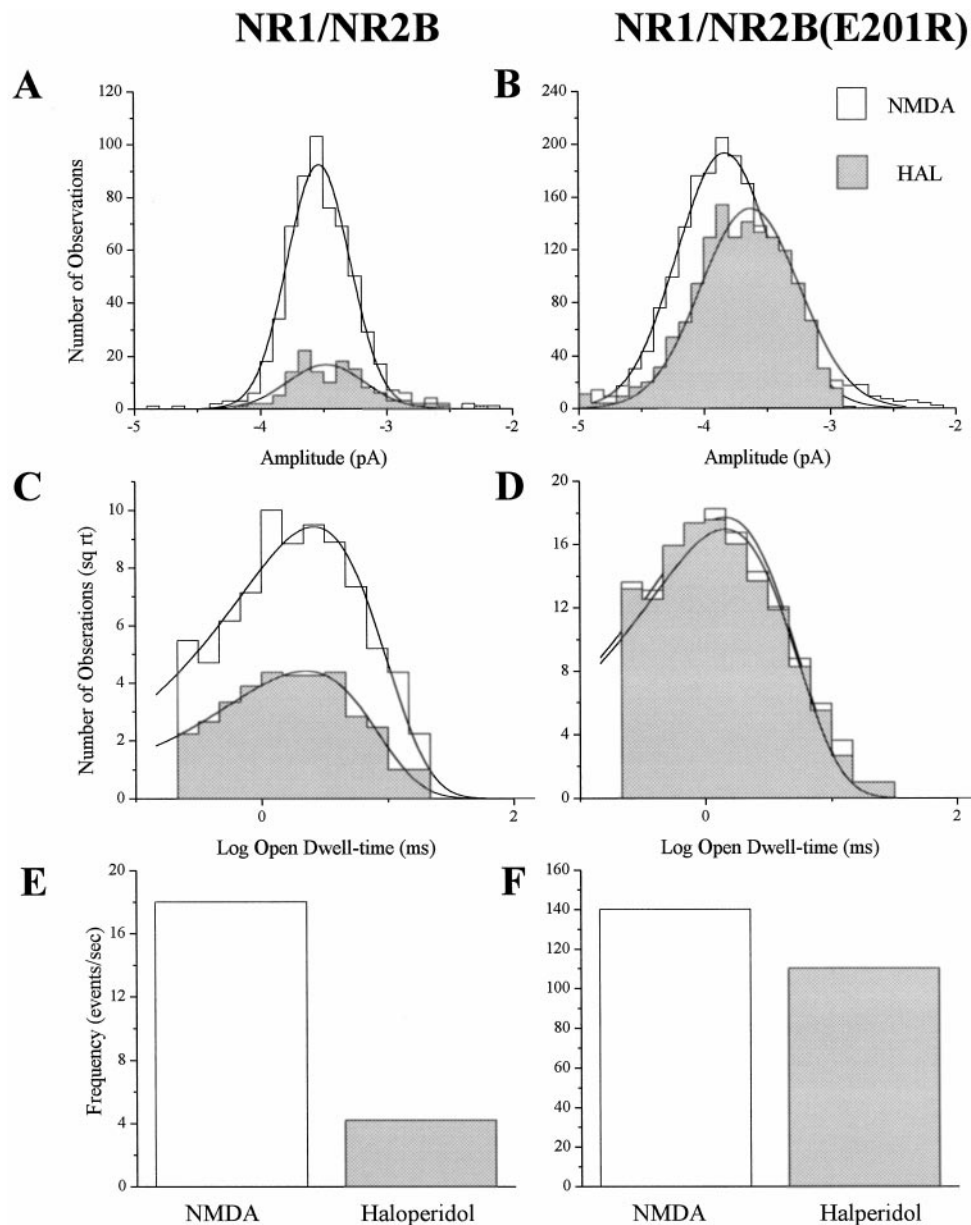


Fig. 4. Haloperidol decreases the frequency of channel opening in NR1/NR2B channels but not in NR1/NR2B(E201R) channels. A and B, amplitude histograms obtained from events elicited by 10 μ M NMDA in the absence or presence of 10 μ M haloperidol. In both subunit combinations, haloperidol slightly decreased the single-channel amplitude. C and D, open dwell-time histograms reveal that this concentration of haloperidol does not alter open time constants in either subunit combination. E and F, 10 μ M haloperidol decreases the frequency of channel opening in a wild-type NR1/NR2B channel but not in a mutant NR1/NR2B(E201R) channel. A, C and E represent data obtained from a single patch excised from an NR1/NR2B-transfected cell; B, D and F represent data obtained from a single patch excised from an NR1/NR2B(E201R)-transfected cell. Similar results were obtained in a total of four wild-type channels and seven mutant channels; compiled data are summarized in table 1.

recording period, with no evidence of patch rundown. Results are expressed as means \pm S.E.M.

Results

We sought to determine the effects of haloperidol and CP101,606 on the single-channel properties of both NR1/NR2B(R337K) and NR1/NR2B(E201R) receptors. Previous studies utilizing 125 I-MK801 binding assays suggested that ifenprodil has an \sim 150-fold higher affinity for wild-type NR1/NR2B receptors than for the arginine 337-mutated receptors (Gallagher *et al.*, 1996). Surprisingly though, attempted single-channel electrophysiological studies on recombinant NR1/NR2B(R337K) receptors expressed in CHO cells seemed to indicate that these mutant receptors were not functional, in spite of their having normal 125 I-MK801 affinity in the binding studies. The lack of functional channels was confirmed by both whole-cell recordings and intracellular Ca^{++} measurements (data not shown). We therefore could only

investigate the NR1/NR2B(E201R) mutant, which has a 10-fold reduced sensitivity to haloperidol when compared with wild-type receptors, as revealed by binding assays (Gallagher *et al.*, 1998).

NMDA (10 μ M)-activated channels were recorded from patches of cells transfected with either NR1/NR2B ($n = 4$) or NR1/NR2B(E201R) ($n = 7$), both in the absence or presence of 10 μ M haloperidol (fig. 3). For the two receptor combinations (fig. 4), haloperidol slightly, albeit significantly, decreased the single-channel amplitudes at a holding voltage of -60 mV. Hence, NR1/NR2B channel amplitudes decreased from -3.7 ± 0.2 in NMDA to -3.4 ± 0.2 pA in NMDA and haloperidol ($P < .05$, paired t test), whereas NR1/NR2B(E201R) channel amplitudes similarly decreased from -3.6 ± 0.1 to -3.4 ± 0.1 pA ($P < .05$, paired t test). Haloperidol also produced a substantial decrease in the frequency of channel opening of wild-type NR1/NR2B channels by 77% but did not significantly decrease their open dwell time

TABLE 1

Single-channel parameters for NR1/NR2B and NR1/NR2B(E201R) receptors in the absence and presence of the two antagonists

	Haloperidol	
	Control	Drug
NR1/NR2B (<i>n</i> = 4)		
Amplitude (pA)	-3.7 ± 0.2	-3.4 ± 0.2*
Open time (msec)	3.0 ± 0.7	2.6 ± 0.5
% ↓ frequency		77 ± 1.5%*
NR1/NR2B(E201R) (<i>n</i> = 7)		
Amplitude	-3.6 ± 0.1	-3.4 ± 0.1*
Open time	3.4 ± 0.5	3.7 ± 0.8
% ↓ frequency		28 ± 16%
	CP101,606	
NR1/NR2B (<i>n</i> = 5)		
Amplitude	-3.7 ± 0.1	-3.6 ± 0.1
Open time	3.9 ± 0.6	2.3 ± 0.3*
% ↓ frequency		67 ± 6.3%*
NR1/NR2B(E201R) (<i>n</i> = 6)		
Amplitude	-3.9 ± 0.1	-3.8 ± 0.1
Open time	3.0 ± 0.4	3.4 ± 0.9
% ↓ frequency		up 9 ± 48%

* Significant difference ($P < .05$) in parameter between control condition *versus* either 10 μM haloperidol or 0.5 μM CP101,606.

(3.0 ± 0.7 control *versus* 2.6 ± 0.5 msec drug). In contrast, NR1/NR2B(E201R) receptors were relatively insensitive to haloperidol, with this drug only slightly decreasing the frequency of channel opening (by 28%). The effects of haloperidol on channel opening frequency were significantly different between wild-type and mutant channels ($P < .05$, unpaired *t* test). Similar to the NR1/NR2B channels, the mean open dwell time of the mutant channel was not altered by haloperidol (3.4 ± 0.5 control *versus* 3.7 ± 0.8 msec drug). Furthermore, the mutation itself did not affect the amplitude or the open dwell time of the channel (table 1).

The actions of CP101,606 on wild-type and haloperidol-insensitive receptors were also examined at the single-channel level. Patches excised from cells transfected with either NR1/NR2B (*n* = 5) or NR1/NR2B(E201R) (*n* = 6) were exposed to 10 μM NMDA in the absence or presence of 0.5 μM CP101,606 (fig. 5). Similar to the results seen in a previous study (Brimecombe *et al.*, 1997), this drug decreased both the open dwell time and the frequency of channel opening of NR1/NR2B channels without altering the amplitude of the currents. The open dwell time of NR1/NR2B channels decreased from 3.9 ± 0.6 msec in NMDA alone to 2.3 ± 0.3 msec in NMDA and CP101,606 ($P < .005$, paired *t* test). When the open dwell-time histograms were best fit with two exponentials, the antagonist did not seem to affect one time constant over the other. In addition, the frequency of channel opening was decreased by 67% in the presence of the drug (fig. 6). In contrast, CP101,606 had no antagonistic effects on NR1/NR2B(E201R) receptors. The open dwell time of the channel was not altered by the drug (3.0 ± 0.4 control *versus* 3.4 ± 0.9 msec drug). Moreover, the frequency of channel opening did not decrease at all but in fact slightly increased in the presence of CP101,606 (fig. 6), opposite to what was seen in the wild-type channels. Therefore, in addition to being less sensitive to haloperidol, NR1/NR2B(E201R) channels are not altered by CP101,606, suggesting that this glutamate residue is critical for the effects of both classes of drugs (table 1).

Discussion

The single-channel data described here suggest that haloperidol and the ifenprodil analog CP101,606 have pharmaco-

logically overlapping sites of action. Haloperidol, at the concentration tested, inhibited recombinant wild-type NR1/NR2B channel activity by decreasing the frequency of channel opening, whereas CP101,606 antagonized these channels by decreasing both the open dwell time and the frequency of channel opening. Haloperidol (3 μM) decreases NMDA receptor activity in young (≤ 10 days *in vitro*) rat cortical neurons by decreasing the frequency of channel opening, whereas higher concentrations (30 μM) decrease both the frequency of channel opening and the open dwell time (Ilyin *et al.*, 1996). This is similar to the observed actions of 3 μM ifenprodil on native NMDA receptors in hippocampal neurons, because this drug inhibits channel activity by decreasing both channel parameters (Legendre and Westbrook, 1991). Here we show that a single amino acid change in the amino terminus of the NR2B subunit (E201R) substantially decreased both the haloperidol and CP101,606 sensitivity of the receptor. These results are in apparent conflict with ^{125}I -MK801 binding results that implicated two separate amino acids as being solely responsible for the two antagonists' actions, *viz.*, arginine 337 for ifenprodil inhibition and glutamate 201 for haloperidol sensitivity (Gallagher *et al.*, 1996; Gallagher *et al.*, 1998). Our data, however, are in support of previous competition binding assays that have demonstrated that haloperidol can inhibit [^3H]ifenprodil binding to rat brain membranes (Coughenor and Cordon, 1997).

There are several possible scenarios that could account for these discrepancies. ^{125}I -MK801 binding assays are sometimes thought to assess interactions with the desensitized state of the receptor, given its high affinity for this ligand and the long time needed to approach equilibrium. A binding assay can also measure receptors that are not present at the cell surface. Consequently, the actual receptor populations studied by both methods may differ and perhaps be modulated in different manners. This could be relevant to this study because ifenprodil, and presumably CP101,606, has a slightly higher affinity with desensitized states of the receptor when compared with the open states (Kew *et al.*, 1996). In addition, ligand-binding assays are performed with much higher concentrations of glycine and glutamate and in the presence of 100 μM spermidine. Because spermidine itself may interact with glutamate 201 (Gallagher *et al.*, 1997), it is possible that the binding of antagonists whose effects are also mediated by this region of NR2B can be altered by the presence of spermidine. This scenario could be similar to the recently noted changes in the median inhibitory concentration (IC_{50}) values of ifenprodil and CP101,606 produced by alterations in extracellular pH (Pahk and Williams, 1997; Zhang *et al.*, 1997).

Another unexpected finding is the absence of electrophysiologically detectable channels in receptors formed by NR1 and the NR2B(R337K) mutant. Cells transfected with this subunit combination have been previously shown to bind ^{125}I -MK801 with high affinity, which is stimulated by polyamines but insensitive to ifenprodil (Gallagher *et al.*, 1996), although the total number of binding sites in these cells is apparently lower (M. J. Gallagher and D. R. Lynch, unpublished observations). Although MK801 binding is activity dependent and enhanced by the same factors that augment physiological responses, agents that block the channel do not always produce identical effects in electrophysiological mea-

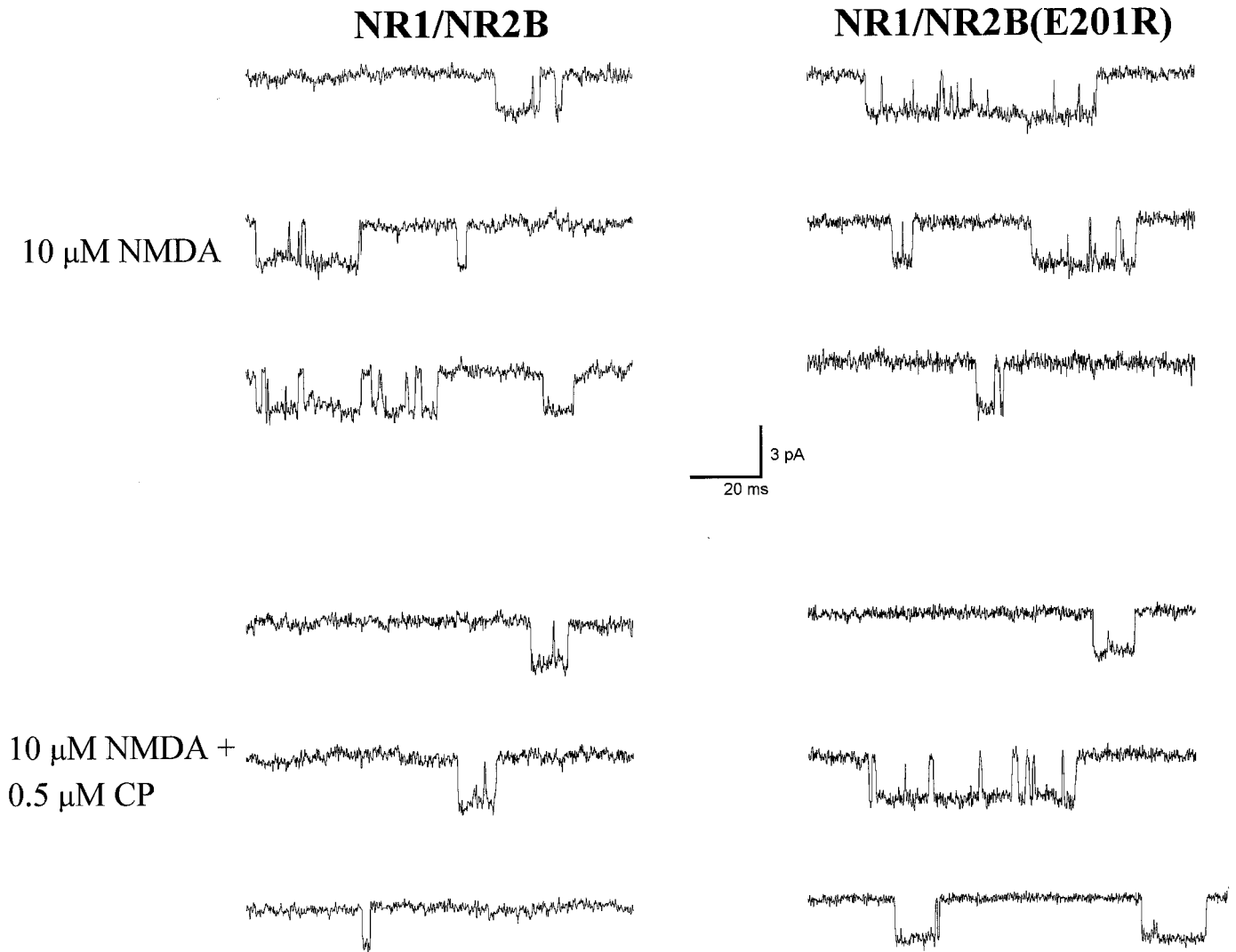


Fig. 5. Representative single-channel events elicited by NMDA in the absence or presence of CP101–606. Single-channel recordings were obtained from outside-out patches excised from cells transfected with either NR1/NR2B or the haloperidol-insensitive receptor NR1/NR2B(E201R). Events were elicited by 10 μ M NMDA in the absence or presence of 0.5 μ M CP101–606.

surements when compared with assays examining the inhibition of MK801 binding (Monaghan and Larsen, 1997). A viable, albeit yet untested, possibility is that the mutation at position 337 produces a permanently desensitized state of the receptor that can still bind MK801. The present results do suggest, however, that the structural requirements for MK801 binding *in vitro* may not translate to functional electrophysiological channels and stress the need to compare results between these techniques with caution.

Experiments demonstrating that haloperidol inhibits NR1/NR2B channel activity by decreasing the frequency of channel opening provides similar results to what was reported for native NMDA receptors in immature cortical neurons (Ilyin *et al.*, 1996), which primarily express the NR1 and NR2B subunits (Zhong *et al.*, 1994). Mutation of glutamate 201 in the amino terminus of the NR2B subunit decreases the ability of haloperidol to inhibit channel activity. In both the wild-type and mutant receptors, haloperidol slightly, but significantly, decreased the single-channel amplitudes. A similar decrease in the amplitude of single-channel responses was seen by Ilyin *et al.* (1996) on native NMDA receptors,

although these authors attributed this effect to a possible measurement error. Although such an effect is suggestive of an unresolved open channel block effect, haloperidol's effect on native NMDA receptors is not voltage dependent (Ilyin *et al.*, 1996). However, the effect on channel amplitude is very small in comparison to the effects of the drug on open dwell time and frequency of channel opening and thus likely accounts for a very minor component of the total block. Furthermore, this slight decrease in amplitude was also present in the mutated NR1/NR2B(E201R) channels, consistent with an action at an additional site, such as the ion channel.

As a final note, glutamate 201 on the NR2B subunit has recently been shown to be important for glycine-independent polyamine stimulation and proton sensitivity of recombinant NR1/NR2B receptors (Gallagher *et al.*, 1997). Mutants with the positively charged arginine in place of the glutamate rendered the receptors insensitive to glycine-independent spermidine stimulation and to proton inhibition. Interestingly, very recent evidence has revealed that CP101,606 may antagonize NR1/NR2B receptors by enhancing proton inhibition, *i.e.*, by shifting the IC_{50} of hydrogen ions from pH 7.4

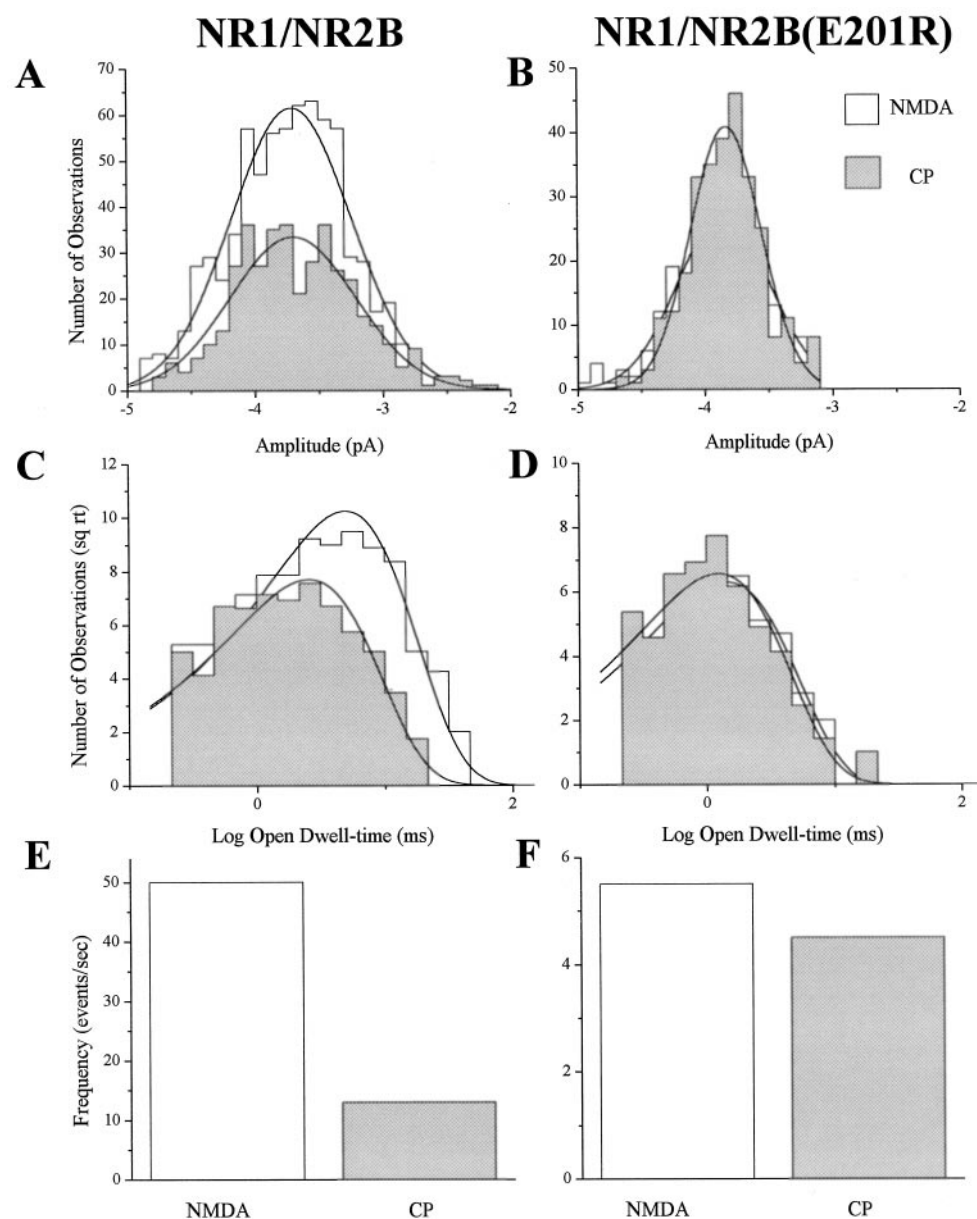


Fig. 6. CP101-606 decreases the open dwell time and the frequency of channel opening of NR1/NR2B channels but not of NR1/NR2B(E201R) channels. A and B, amplitude histograms obtained from events elicited by 10 μ M NMDA in the absence or presence of 0.5 μ M CP101-606. This antagonist does not alter the single-channel amplitude of either subunit configuration. C and D, CP101,606 decreases the open time constant of NR1/NR2B channels but not that of the haloperidol-insensitive channel. E and F, CP101,606 also decreases the frequency of channel opening in wild-type channels, whereas NR1/NR2B(E201R) channels are unaffected. A, C and E represent data obtained from a single patch excised from an NR1/NR2B-transfected cell; B, D and F represent data obtained from a single patch excised from an NR1/NR2B(E201R)-transfected cell. Similar results were obtained for five wild-type channels and six mutant channels; compiled data are summarized in table 1.

to 9.3 (Zhang *et al.*, 1997). Other investigators have demonstrated that mutation of aspartate 669 in the NR1 subunit, located in the extracellular M3 to M4 loop, abolishes glycine-independent polyamine stimulation, reduces proton inhibition and decreases ifenprodil block in NR1/NR2B receptors (Kashiwagi *et al.*, 1996). Clearly then, many residues within the NMDA receptor protein are likely participating in the formation of the binding sites for all of these different modulators, including both ifenprodil and haloperidol.

Acknowledgments

We thank W. K. Potthoff, K. A. Hartnett and S. R. Arden for helpful suggestions and technical assistance; S. Nakanishi, P. Seeburg and M. Chalfie for plasmids; and W. F. White for CP101,606.

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