

# Phenylethanolamines inhibit NMDA receptors by enhancing proton inhibition

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The phenylethanolamines, ifenprodil and CP-101,606, are NMDA receptor antagonists with promising neuroprotective properties. In recombinant NMDA receptors expressed in *Xenopus* oocytes, we found that these drugs inhibit NMDA receptors through a unique mechanism, making the receptor more sensitive to inhibition by protons, an endogenous negative modulator. These findings support a critical role for the proton sensor in gating the NMDA receptor and point the way to identifying a context-dependent NMDA receptor antagonist that is inactive at physiological pH, but is a potent inhibitor during the acidic conditions that arise during epilepsy, ischemia and brain trauma.

The neurotoxic actions of glutamate acting at the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor may contribute to the neuronal degeneration associated with several neurological diseases<sup>1–3</sup>. Of the many regulatory sites on NMDA receptors, the proton sensor has received little attention during the search for neuroprotective strategies. NMDA receptors expressed by forebrain and cerebellar neurons are inhibited by protons with an IC<sub>50</sub> value that corresponds to pH 7.3 (refs 4–8). Thus, at physiological pH, these NMDA receptors are tonically inhibited by protons, suggesting that small changes in interstitial pH could alter NMDA receptor function. Interstitial pH undergoes multiphasic changes during normal synaptic transmission<sup>9</sup>, and much larger changes during intense seizure activity and ischemia, during which pH levels can fall by 0.2 to more than 1.0 pH units<sup>9–11</sup>. Because neuronal injury during both seizures and ischemia is associated with glutamate release and NMDA receptor activation, acidification of the extracellular space during these conditions would be expected to limit the extent of neurotoxicity, assuming that acid conditions persist during the period in which glutamate levels are elevated. In support of this hypothesis, decreasing extracellular pH to a level observed during ischemia reduces the contribution of NMDA receptors to neuronal death in cortical cultures<sup>4,12</sup>.

The phenylethanolamines, exemplified by ifenprodil, are allosteric NMDA receptor antagonists<sup>13</sup> with chemical structures unlike any of the competitive or uncompetitive antagonists (Fig. 1a). High-affinity inhibition by ifenprodil depends critically on the presence of the NR2B subunit<sup>14,15</sup>. The NR2B subunit can be coimmunoprecipitated with the NR1 subunit in the adult cortex and thus is a component of NMDA receptors in this region<sup>16,17</sup>. Ifenprodil is neuroprotective against glutamate excitotoxicity in cell culture<sup>18</sup> and in animal models of focal cerebral ischemia<sup>19,20</sup>. Together, these findings strongly suggest that NMDA receptors containing the NR2B subunit contribute to excitotoxic damage.

Several ifenprodil analogs are currently in clinical evaluation, but their mechanisms of action are unclear. Ifenprodil has been

variously proposed to stabilize a desensitized state of the receptor<sup>21</sup>, to block the spermine binding site<sup>22</sup>, to allosterically inhibit glycine binding<sup>14,23</sup>, to be a weak open-channel blocker<sup>14</sup>, or to promote transitions to a nonconducting state of the channel<sup>23</sup>. Ifenprodil stabilizes an agonist-bound state of the receptor that has low open probability<sup>24</sup>, but the mechanism for this effect is unknown. Unfortunately, the clinical development of ifenprodil has been prevented because it also blocks alpha-adrenergic, 5HT<sub>1A</sub>, 5HT<sub>2</sub>, 5HT<sub>3</sub> and sigma receptors and calcium channels<sup>25,26</sup>. The poor selectivity of ifenprodil has led to exploration of other phenylethanolamine derivatives with higher selectivity, such as Ro 25-6981 (ref. 27), Ro 8-4304 (ref. 28) and CP-101,606 (Fig. 1a). The latter is a noncompetitive NMDA receptor antagonist<sup>29</sup> which lacks the alpha-adrenergic and serotonergic affinity of ifenprodil as well as the psychomotor stimulant effects of other NMDA receptor antagonists.

Our data indicate that both ifenprodil and CP-101,606 inhibit NMDA receptors through a similar and unique mechanism—shifting the pK<sub>a</sub> of the proton sensor to a more alkaline level, which would enhance tonic inhibition at physiological pH. Thus, the phenylethanolamines seem to inhibit NMDA receptors by increasing the sensitivity of the receptor to negative modulation by protons. Our findings lead to a model supporting a key role for the proton sensor in gating of the NMDA receptor channel; they also suggest a strategy to identify neuroprotective agents with a high therapeutic index. Preliminary accounts of this work have previously appeared (Mott *et al.*, *Soc. Neurosci. Abstr.* 1996, 1998; Zhang *et al.*, *Soc. Neurosci. Abstr.* 1997).

## Results

### SIMILARITY IN ACTION OF CP-101,606 AND IFENPRODIL

Ifenprodil is a voltage-independent and subunit-selective blocker of NMDA receptors<sup>13,30</sup>. In contrast, the only published information on CP-101,606 concerns its neuroprotective effects<sup>29,31</sup>. Therefore, we characterized the action of CP-101,606 on NMDA receptors and compared inhibition produced by this antagonist to that pro-

duced by ifenprodil. CP-101,606 inhibited the steady-state current induced by saturating concentrations of NMDA (100  $\mu$ M) and glycine (10  $\mu$ M) in oocytes injected with RNA encoding the NR1-1a and NR2B receptor subunits (Fig. 1b). (NR1-1a is a splice variant of the NR1 subunit that lacks exon 5.) At pH 7.5, CP-101,606 inhibited NMDA currents with an  $IC_{50}$  of 39 nM (95% confidence interval (c.i.), 30–51 nM) that was 4.3 times more potent than ifenprodil ( $IC_{50}$  = 167 nM; 95% c.i., 114–245 nM; Fig. 1c). The  $IC_{50}$  for inhibition of NR1-1a/NR2B receptors by ifenprodil was identical to that measured in cortical neurons<sup>24</sup> (170 nM). Neither ifenprodil nor CP-101,606 completely inhibited NMDA currents, with the maximal inhibition of NR1-1a/NR2B receptors being 79–91%.

At pH 7.5, CP-101,606 produced only minimal inhibition of NMDA receptors containing the NR2A, NR2C or NR2D subunits (Fig. 1c), similar to ifenprodil<sup>14,15</sup>. This indicates that high-affinity inhibition by CP-101,606 at physiological pH also depends critically on the presence of the NR2B subunit. As reported for ifenprodil<sup>14</sup>, inhibition of NR1-1a/NR2B current by CP-101,606 was voltage independent ( $n = 7$  oocytes, data not shown). The potency of CP-101,606 was the same in 0.3  $\mu$ M ( $n = 4$  oocytes) and 10  $\mu$ M glycine ( $n = 8$  oocytes, data not shown), arguing against an interaction with the glycine binding site<sup>32</sup>. The block was noncompetitive but was associated with a small increase in NMDA potency, as shown with cortical neurons for ifenprodil<sup>24</sup> (Fig. 1d, legend). Thus, CP-101,606 and ifenprodil seem to act on NMDA receptors through a similar mechanism.

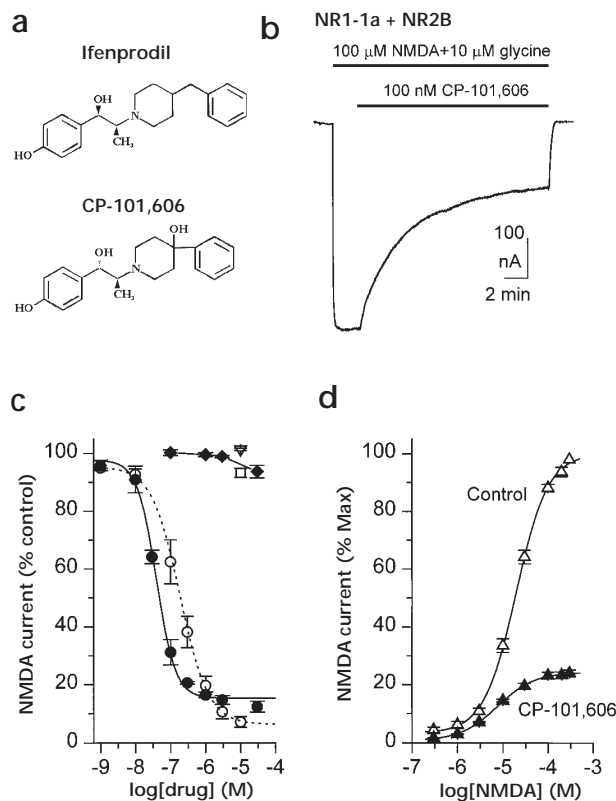
#### PHENYLETHANOLAMINES ACT ON THE PROTON SENSOR

Like phenylethanolamines, protons produce a noncompetitive, voltage-independent inhibition of NR1-1a/NR2B receptors. To determine whether the mechanism of action of phenylethanolamines is linked to that of protons, we examined the drugs' effect on proton inhibition of recombinant NMDA receptors. In the presence of increasing concentrations of either CP-101,606 or ifenprodil, NR1-1a/NR2B receptors became progressively more sensitive to protons (Fig. 2a and b). For example, the  $IC_{50}$  for proton inhibition of the receptor was shifted from pH 7.4  $\pm$  0.03 to pH 7.8  $\pm$  0.07 in 30 nM CP-101,606 ( $p < 0.01$ ). In the presence of this drug, a higher fraction of receptors would seem to be protonated, and thus inhibited, at any given pH. Plots of the pH causing 25% inhibition of the receptor at different drug concentrations revealed that CP-101,606 produced a greater shift in proton sensitivity than did ifenprodil for a given concentration of drug (Fig. 2c), mirroring the relative potency of these two drugs at pH 7.5 (Fig. 1c). These results suggest that both CP-101,606 and ifenprodil inhibit NMDA current by shifting the  $pK_a$  of the proton sensor on the NMDA receptor to more alkaline values.

These results cannot be explained by a pH-dependent change in the proportion of ionized species of CP-101,606, because the  $pK_a$ s for the drug are beyond the range of pH studied here. The structural requirements of NMDA receptor inhibition by zinc and by protons are similar<sup>33</sup>, raising the possibility that phenylethanolamines may inhibit by enhancing the affinity of the NMDA receptor for zinc. However, removal of trace levels of zinc from our perfusion solution by the addition of a low concentration of the metal chelator EDTA (10  $\mu$ M) did not alter ifenprodil potency ( $n = 7$ , data not shown). Thus, phenylethanolamine inhibition is not mediated by the inhibitory zinc site.

#### PHENYLETHANOLAMINES ENHANCE PROTON INHIBITION

We tested four predictions of the hypothesis that the mechanisms of NMDA receptor inhibition by protons and phenylethanolamines are linked. First, inhibition by phenylethanolamines should be

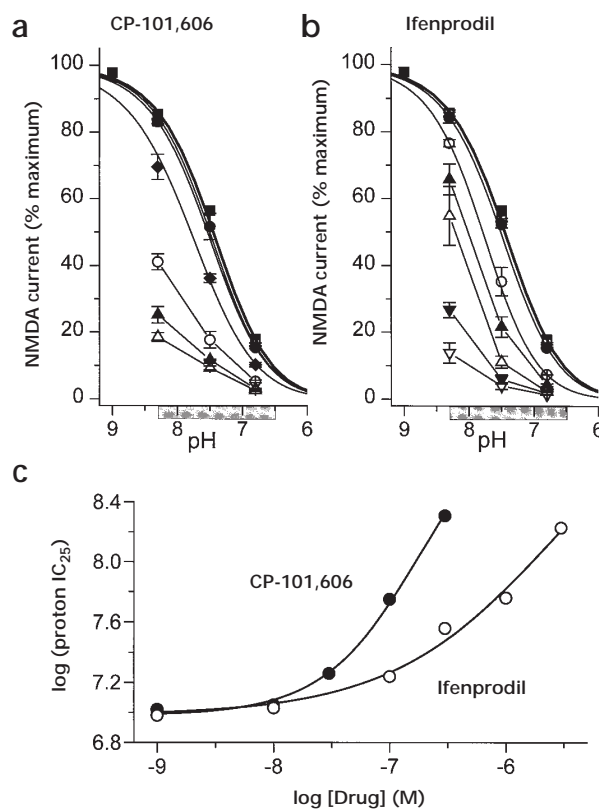


**Fig. 1.** CP-101,606 and ifenprodil inhibit NMDA receptors in a subunit selective and noncompetitive fashion. **(a)** The structures of ifenprodil and CP-101,606. **(b)** Representative current recorded from an oocyte showing inhibition of NR1-1a/NR2B currents at pH 7.5 by 100 nM CP-101,606. **(c)** Concentration-inhibition curves for antagonism of NMDA receptor currents by CP-101,606 and ifenprodil at pH 7.5 ( $n = 3$ –12 oocytes at each concentration). (●), CP-101,606 versus NR1-1a/NR2B receptors. (○), ifenprodil versus NR1-1a/NR2B receptors. (◆), (□) and (△), CP-101,606 versus NR1-1a/NR2A, /NR2C and /NR2D receptors, respectively. **(d)** Concentration-response curves were obtained for activation of NR1-1a/NR2B receptors by NMDA in the absence and presence of 100 nM CP-101,606, as shown (10  $\mu$ M glycine;  $n = 7$ –8 oocytes for each curve). The  $EC_{50}$  for NMDA was 20  $\mu$ M (95% c.i., 17–22  $\mu$ M) in control and 8  $\mu$ M (95% c.i., 6–11  $\mu$ M) in the presence of CP-101,606. All values are expressed as a percentage of the current at 300  $\mu$ M NMDA in the absence of CP-101,606. The current amplitude at 300  $\mu$ M NMDA in the absence of drug was within 1% of the fitted maximum, indicating that this NMDA concentration maximally activated these receptors.

completely overcome when proton concentration is sufficiently reduced. Second, mutations that reduce proton inhibition should proportionately weaken inhibition produced by phenylethanolamines. Third, spermine and the presence of exon 5 in the NR1 subunit (NR1-1b), both of which potentiate NMDA currents by relieving proton inhibition of the receptor<sup>34</sup>, should oppose phenylethanolamine inhibition. Finally, lowering the ionic strength of the perfusion solution, which reduces shielding of the proton sensor<sup>34</sup>, should potentiate the effects of spermine and exon 5 on phenylethanolamine inhibition.

If ifenprodil inhibits NMDA receptors by potentiating proton inhibition, its effect should disappear at sufficiently alkaline

**Fig. 2.** Phenylethanolamines enhance proton inhibition of the NMDA receptor. Proton inhibition curves of NR1-1a/NR2B receptors ( $n = 5-8$ ) at increasing concentrations of either CP-101,606 (**a**) or ifenprodil (**b**). The drug concentrations used were 1 nM ( $\square$ ), 10 nM ( $\bullet$ ), 30 nM ( $\blacklozenge$ ), 100 nM ( $\circ$ ), 300 nM ( $\blacktriangle$ ), 1  $\mu$ M ( $\triangle$ ), 3  $\mu$ M ( $\blacktriangledown$ ), 10  $\mu$ M ( $\nabla$ ). In the absence of antagonist ( $\blacksquare$ ), protons inhibited the receptor with an  $IC_{50}$  corresponding to pH 7.4. NMDA current amplitude at each pH in the presence of the phenylethanolamine was normalized relative to the amplitude of the current at that pH in the absence of the drug. Data were then fit to a logistic curve only if the current at pH 8.3 was  $\geq 70\%$  of control maximum. The control proton inhibition curve was constructed by normalizing the current amplitude at each pH to that at pH 7.5. These data were fit to a logistic curve (with constrained minimum of 0%), and the extrapolated maximum of this curve was then used to renormalize the data. In this figure and in Fig. 3a, the shaded box on the abscissa indicates the typical physiological and pathophysiological range of pH values. (**c**) A plot of the pH causing 25% inhibition at the indicated concentrations of CP-101,606 or ifenprodil. Note that the curve for ifenprodil is right shifted compared to that of CP-101,606, indicating that at each tested concentration CP-101,606 produced a stronger alkaline shift in proton inhibition than did ifenprodil.

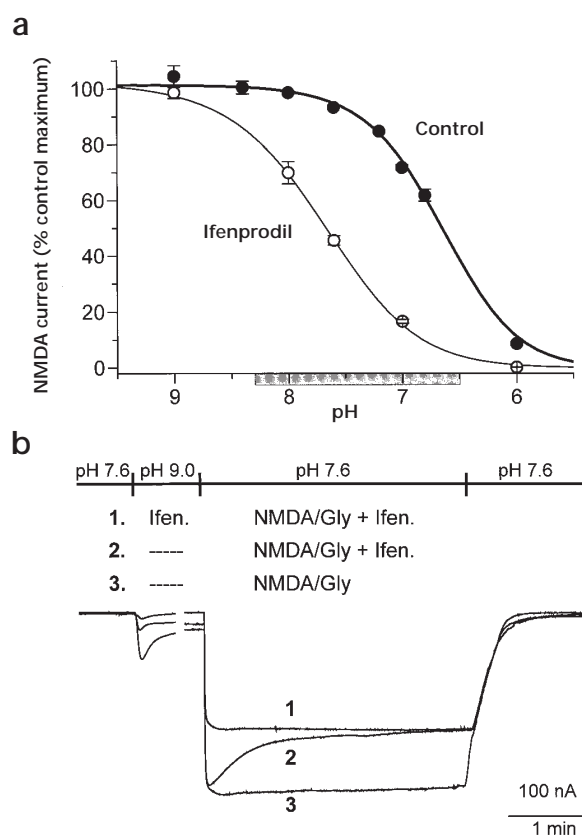


pH. To test this, we used a NR1-1a mutant, D669E, whose pKa for proton block is 6.61, compared with 7.42 for the wild-type subunit. This mutant's reduced proton sensitivity allowed us to test whether ifenprodil was capable of producing inhibition at pH levels that were sufficiently alkaline to remove all tonic proton inhibition. The inhibition produced by 3  $\mu$ M ifenprodil was eliminated at pH 9.0 in these receptors (Fig. 3a). To be certain that the loss of inhibition by ifenprodil at pH 8.0 and 9.0 was not due to a change in ionization state of the drug, we showed that the drug had no effect even when its concentration was increased to compensate for the pH increase, thereby maintaining a constant concentration of the fully ionized drug (see Fig. 3 legend). The finding that inhibition by ifenprodil could be completely overcome at sufficiently low proton concentration supports the proposed hypothesis.

Is ifenprodil's loss of activity at pH 9.0 (Fig. 3a) due to a loss of binding to the NMDA receptor? We took advantage of the slow onset of ifenprodil block compared with the fast onset of agonist action to show that this is not the case (Fig. 3b). Oocytes were equilibrated with 1  $\mu$ M ionized ifenprodil at pH 9.0, then agonists were added as the pH was simultaneously dropped to 7.6. The block was fully developed immediately, indicating that ifenprodil had bound to the receptor at pH 9.0. If ifenprodil were not bound at pH 9.0, then a gradually developing block would have been observed, as occurred when oocytes were equilibrated at pH 9.0 without ifenprodil and then treated with 1  $\mu$ M ionized ifenprodil together with agonists at pH 7.6. Similar results were obtained in 10 cells. This experiment shows that the loss of ifenprodil inhibition at pH 9.0 is not due to a loss of binding. Rather, these results suggest that phenylethanolamines increase the inhibitory coupling of the proton sensor to the channel-gating mechanism by increasing the likelihood that the sensor is protonated.

Several mutations in the NR1 subunit reduce the degree of proton inhibition of the receptor<sup>33,35-37</sup>. If ifenprodil acts by potentiating proton inhibition, then mutations that weaken the influence of the proton sensor on channel gating should to the same extent weaken the potency of ifenprodil. To test this structural prediction, we constructed concentration-inhibition curves for both protons and ifenprodil against NR1-1a/NR2B and a series of nine NR1-1a mutants co-expressed with NR2B. These mutations are scattered throughout the NR1-1a sequence (Fig. 4d). In each oocyte, four or five concentrations of ifenprodil (at pH 7.5), or six concentrations of protons, were tested against pulses of NMDA plus glycine, with washout between antagonist treatments (Fig. 4a). Two agonist pulses were delivered for each concentration of antagonist to ensure that equilibrium block had been reached.  $IC_{50}$ s for protons and ifenprodil were then calculated from fitted curves constructed for 8-15 oocytes per mutation (Fig. 4b). There was an excellent correlation ( $r = .994$ ) between the  $IC_{50}$  for protons and ifenprodil (Fig. 4c) in this set of mutations, which strongly supports the hypothesis.

We then examined the effects of spermine or exon 5 of the NR1 subunit on phenylethanolamine inhibition of NMDA currents. We first tested their effects at pH 6.8 in a solution of low ionic strength, in which relief from proton inhibition caused by spermine or exon 5 is strong. Spermine (100  $\mu$ M) produced a 44-fold rightward shift in the concentration-inhibition curve for CP-101,606, shifting the  $IC_{50}$  from 28 nM (95% c.i., 13-62 nM) in control to 1240 nM (95% c.i., 761-2010 nM) in the presence of spermine (Fig. 5a). The presence of exon 5 slightly decreased the potency of CP-101,606 inhibition, shifting the  $IC_{50}$  1.9-fold rightward to 52 nM (95% c.i., 37-72 nM; Fig. 5a). Spermine was more effective than



**Fig. 3.** Alkaline pH reduces ifenprodil inhibition but not ifenprodil binding. **(a)** Complete proton inhibition curve for NMDA receptors consisting of the NR1-1a(D669E) mutant and NR2B, in the presence (○,  $n = 5-17$  oocytes per point) and absence (●,  $n = 3-21$  oocytes) of 3  $\mu\text{M}$  ionized ifenprodil. Inhibition by ifenprodil was completely reversed at pH 9.0. To compensate for reduction in drug ionization at alkaline pH, at pH 8.0 and 9.0, the bath concentration of ifenprodil was set to 3.7  $\mu\text{M}$  and 10  $\mu\text{M}$ , respectively, resulting in 3.0  $\mu\text{M}$  ionized drug in each case. **(b)** Comparison of the time course of block of NR1-1a(D669E)/NR2B receptors by 1  $\mu\text{M}$  ionized ifenprodil either preapplied at pH 9.0 (trace 1) or applied simultaneously with 300  $\mu\text{M}$  NMDA plus 20  $\mu\text{M}$  glycine at pH 7.6 (trace 2). In trace 2, NMDA receptor activation is limited by the speed of solution exchange, so that inhibition by ifenprodil developed over a period of about 2 min. If the loss of ifenprodil inhibition at pH 9.0 in (a) had been caused by loss of ifenprodil binding at this pH, traces 1 and 2 should be identical; in contrast, block was fully developed at pH 9.0, as demonstrated by the absence of a slowly developing block in trace 1. Trace 3 shows the response to 300  $\mu\text{M}$  NMDA plus 20  $\mu\text{M}$  glycine applied in the absence of antagonist at pH 7.6. To ensure a constant concentration of ionized drug, the concentration of ifenprodil was increased to 3.3  $\mu\text{M}$  at pH 9.0. Small proton leak currents are visible when the solution was switched from pH 7.6 to 9.0. Proton leak currents are of variable size and result in small shifts in the baseline current, as shown here. No attempt was made to compensate for or offset leak currents. The gap in the traces is approximately three minutes. The steady-state inhibition of NMDA currents by ifenprodil was identical when assessed at pH 7.6, regardless of whether ifenprodil was pre-applied at pH 9.0 or co-applied with NMDA at pH 7.6. All three traces were from the same cell.

exon 5 both in opposing CP-101,606 inhibition (Fig. 5a) and in shielding the proton sensor<sup>34</sup>.

As reported<sup>34</sup>, the presence of exon 5 reduced spermine potentiation of NR1-1b/NR2B receptors at pH 6.8 by  $94 \pm 1\%$  ( $n = 9$ , data not shown). However, despite reduced spermine potentiation, the effect of spermine on CP-101,606 inhibition of these receptors was markedly enhanced. Thus, spermine lowered the potency of CP-101,606 at exon-5-containing receptors, shifting the  $\text{IC}_{50}$  169-fold to the right to a value of 4740 nM (95% c.i., 527–42,600 nM; Fig. 5a). This represented a 3.8-fold greater shift in the  $\text{IC}_{50}$  than that produced by spermine on receptors lacking exon 5. Thus, exon 5 and spermine acted synergistically on the receptor to reduce inhibition by CP-101,606. Because spermine and exon 5 occlude each other's actions on the proton sensor, their synergistic ability to reduce ifenprodil potency cannot be due to a greater combined effect on the proton sensor itself, but instead may reflect a reduction of phenylethanolamine binding. The ability of proton-site modulators to hinder phenylethanolamine inhibition further links the mechanism of action of these drugs to the proton site of the NMDA receptor.

At normal ionic strength, neither spermine (100  $\mu\text{M}$ ) nor exon 5 alone increased the  $\text{IC}_{50}$  for inhibition by CP-101,606. However, when spermine was applied to NR1-1b/NR2B (exon 5 containing) receptors, the combination significantly reduced the potency of CP-101,606 (Fig. 5b).

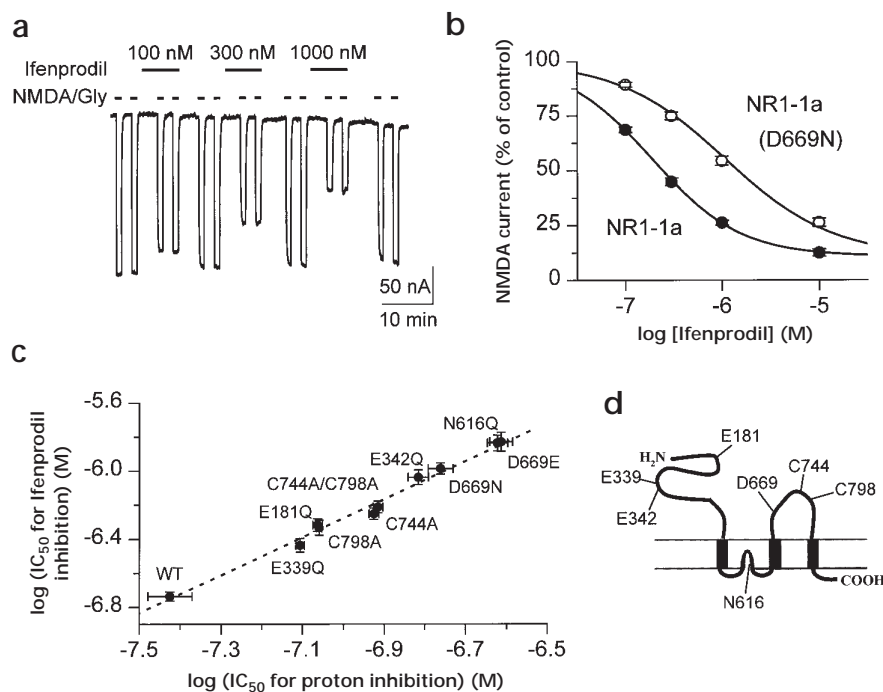
#### IFENPRODIL AND CP-101,606 DIFFER IN THEIR pH DEPENDENCE

The above results indicate that phenylethanolamines potentiate proton inhibition. Does low pH potentiate inhibition by phenylethanolamines? We examined the effect of changes in extracellular pH on both CP-101,606 and ifenprodil inhibition

of NMDA currents in oocytes injected with NR1-1a/NR2B receptors. Decreasing the pH from 8.3 to 6.8 significantly increased the degree of inhibition by 300 nM ifenprodil (Fig. 6a). The inhibitory potency of both CP-101,606 and ifenprodil was accordingly increased (Fig. 6b and c; see also ref. 38). However, over this pH range, ifenprodil was more sensitive than CP-101,606 to changes in proton concentration. In particular, lowering the pH from 7.5 to 6.8 increased ifenprodil potency 2.4-fold, but had no effect on the inhibitory potency of CP-101,606 (Fig. 6b and d).

In heterodimeric NMDA receptors, different degrees of proton sensitivity are conferred by the four NR2 subunits<sup>34</sup>. As judged by the ratio of NMDA-induced current at pH 6.8 and pH 7.5, NR2A and NR2B are the most proton sensitive, whereas NR2C-containing receptors are the least sensitive. Compared to pH 7.5, current through NR2A- and NR2B-containing receptors was inhibited by  $80 \pm 1\%$  and  $70 \pm 4\%$ , respectively, when the pH was lowered to pH 6.8. In contrast, lowering the pH to 6.8 inhibited only  $30 \pm 10\%$  of the NR2C current and  $52 \pm 6\%$  of the NR2D current. At pH 7.5, high-affinity inhibition by phenylethanolamines requires the NR2B subunit<sup>30</sup> (Fig. 1c), and all previous studies of subunit selectivity for inhibition by phenylethanolamines were done at physiological pH. At pH 6.8, however, in addition to inhibiting NR2B-containing receptors, CP-101,606 inhibited NR2A-containing receptors, although with very low potency (Fig. 6e). These results indicate that at acidic pH levels, the NR2B subunit is not an absolute requirement for phenylethanolamine inhibition.

Changes in ionization of these drugs at the different pHs could potentially complicate the interpretation of these results by altering the effective concentration of antagonist. However, based on the  $\text{pK}_a$  values for CP-101,606 (9.07 for the tertiary

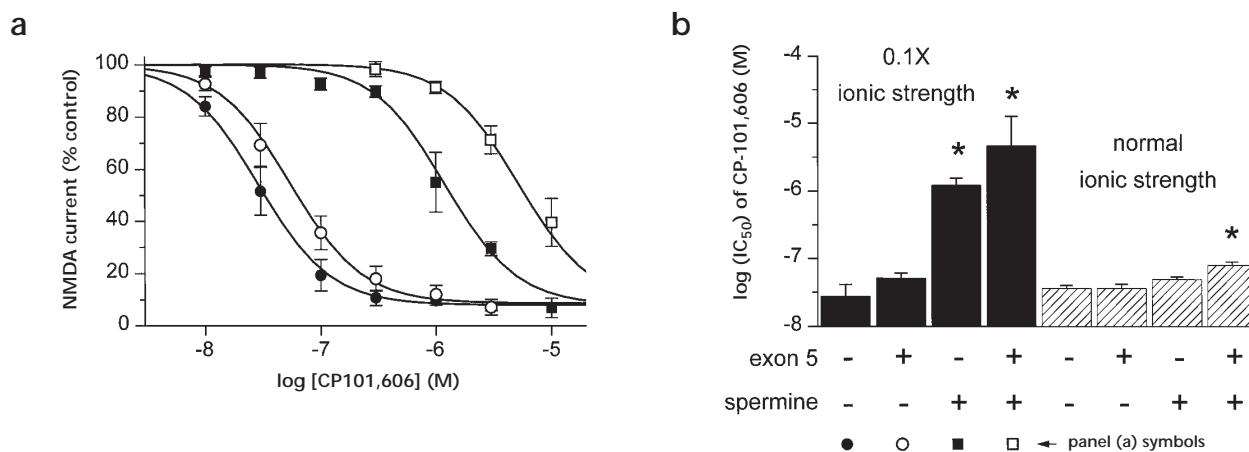


**Fig. 4.** Equivalent effect of NR1-1a mutations on inhibition by protons and ifenprodil. **(a)** Sample NR1-1a(D669N)/NR2B currents evoked by 100  $\mu$ M NMDA plus 10  $\mu$ M glycine and the indicated concentrations of ifenprodil (membrane voltage,  $-20$  mV). **(b)** Concentration-inhibition curves constructed from data such as that shown in (a) for wild-type NR1-1a ( $\bullet$ ,  $n = 13-17$ ) and the D669N mutant of NR1-1a ( $n = 8$ ), both coexpressed with NR2B. **(c)** The  $IC_{50}$  for proton inhibition plotted against the  $IC_{50}$  for ifenprodil inhibition, in NMDA receptors formed from NR2B plus wild-type NR1-1a or one of a series of nine NR1-1a mutants, as indicated ( $n = 8-15$  oocytes for each receptor). A linear regression fit to these points revealed an excellent correlation ( $r = 0.994$ ).  $IC_{50}$ s were calculated from curves such as those shown in (b). **(d)** A schematic diagram showing the location of each mutation in relation to the four membrane domains of NR1-1a.

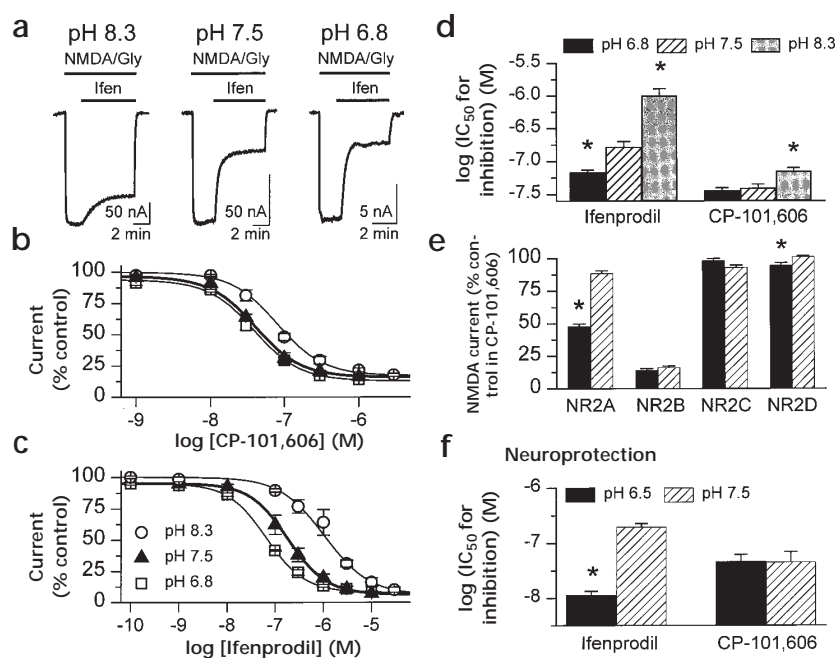
nitrogen group, 9.50 for the phenolic hydroxyl), the percentage of fully protonated antagonist changes from 86% to 99% as the pH is reduced from 8.3 to 6.8. Similarly, ifenprodil has two ionizable groups, one with  $pK_a$  of 8.63 (tertiary nitrogen) and the other of 9.67 (phenolic hydroxyl), indicating that fully ionized ifenprodil changes from 68% to 98% of the total over the tested pH range. The direction of these changes makes it unlikely that the protonation state of either CP-101,606 or ifenprodil could explain the observed changes in potency of these antagonists over the pH range used in this study.

#### NEUROPROTECTION IS ENHANCED AT ISCHEMIC pH

All of the above results were obtained using recombinant NMDA receptors. To determine whether phenylethanolamine inhibition of native NMDA receptors is similarly increased at low pH, we measured the potency of ifenprodil and CP-101,606 as blockers of NMDA-induced excitotoxicity in primary cultures of rat cerebral cortex. Under our conditions, pH 6.5 itself afforded some neuroprotection as compared with pH 7.5, because the maximal lactate dehydrogenase release produced by NMDA was reduced from  $30 \pm 4\%$  ( $n = 16$ ) of the total in the culture well at pH 7.5 to



**Fig. 5.** Spermine and exon 5 synergistically reduce inhibition by CP-101,606. **(a)** Concentration-inhibition curves for inhibition of NR2B-containing NMDA receptors at pH 6.8 by CP-101,606 in low (0.1x) ionic strength under the following conditions: exon-5-lacking NR1-1a receptors ( $\bullet$ ); exon-5-containing NR1-1b receptors ( $\circ$ ); exon-5-lacking receptors in the presence of 100  $\mu$ M spermine ( $\blacksquare$ ); exon 5-containing receptors in the presence of 100  $\mu$ M spermine ( $\square$ ). **(b)**  $IC_{50}$  for CP-101,606 inhibition in each condition tested, for low ionic strength (black bars) and normal ionic strength (hatched bars). An asterisk indicates a significant increase in the  $IC_{50}$  compared to that in the absence of both exon 5 and spermine ( $p < 0.05$ , ANOVA with post-hoc Bonferroni test).



**Fig. 6.** Ifenprodil and CP-101,606 differ in their pH dependence. **(a)** Inhibition by 300 nM ifenprodil at the three indicated pHs, all in the same oocyte. Concentration–inhibition curves for **(b)** CP-101,606 and **(c)** ifenprodil at three different pH levels. The protocol shown in **(a)** was used to construct these concentration–inhibition curves. The pH levels used in these experiments reflect extremes of the physiological range. **(d)** Bar graph comparing the potency of CP-101,606 and ifenprodil at the three pHs tested. Note that reducing the pH from pH 7.5 to pH 6.8 produced a 2.8-fold increase in ifenprodil potency but had little effect on the potency of CP-101,606. Asterisks indicate a significant difference from pH 7.5 ( $p < 0.01$ , ANOVA with post-hoc Bonferroni test). **(e)** Effect of NR2 subunit on inhibition by CP-101,606 at two pHs. The percent inhibition produced by 10  $\mu$ M CP-101,606 (1  $\mu$ M for NR2B) was measured at pH 7.5 (hatched bars) and pH 6.8 (solid bars) from oocytes injected with NR1-1a plus the NR2 subunit indicated ( $n = 3$ –8 for each condition tested). Asterisks indicate a significant increase in inhibition by CP-101,606 ( $p < 0.01$ , ANOVA with post-hoc Bonferroni test). **(f)** The neuroprotective potency of ifenprodil, but not CP-101,606, is increased at acidic pH. Twenty independent cortical culture preparations were tested. The  $IC_{50}$ s for ifenprodil are 11 nM (95% c.i., 8.1–16 nM) at pH 6.5 and 199 nM (95% c.i., 147–269 nM) at pH 7.5;  $IC_{50}$ s for CP-101,606 are 47 nM (95% c.i., 25–87 nM) at pH 6.5 and 46 nM (95% c.i., 19–112 nM) at pH 7.5. Asterisk indicates a significant increase in ifenprodil potency at pH 6.5 ( $p < 0.01$ , unpaired  $t$ -test).

$17 \pm 5\%$  ( $n = 21$ ) at pH 6.5 (see also refs 4, 12). In agreement with findings from recombinant receptors, in cultures that showed excitotoxicity mediated by NMDA receptors, ifenprodil was about 18 times more potent as a neuroprotectant at pH 6.5 than at pH 7.5, but the potency of CP-101,606 was identical at the two pHs (Fig. 6f). This finding suggests that the neuroprotective effects of ifenprodil, but not CP-101,606, are enhanced at the acidic pH values typical of ischemic tissue.

### Discussion

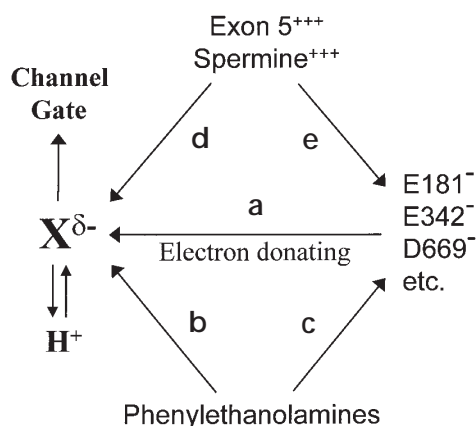
We conclude that inhibition of NMDA receptors by phenylethanolamines occurs by enhancing inhibition by protons that act as an endogenous negative modulator. This proposed mechanism is supported by four lines of evidence. First, in the NR1-1a(D669E) mutant, inhibition by CP-101,606 could be completely abolished at sufficiently alkaline pH, as expected if the drug acted through the proton sensor. Second, both ifenprodil and CP-101,606 shifted the proton-inhibition curve to the left. Third, nine different mutations of the NR1-

1a subunit caused shifts in the potency of ifenprodil that were quantitatively identical to the corresponding shifts in the potency of protons, pointing to common structural elements controlling the actions of both inhibitors. Finally, the potency of CP-101,606 as an NMDA receptor antagonist was reduced by the combination of spermine and exon 5 of the NR1 subunit, both of which weaken proton inhibition by shielding the proton sensor<sup>34</sup>. This effect of spermine and exon 5 was greatly enhanced at low ionic strength, as expected from their proposed electrostatic shielding mechanism<sup>34</sup>. An opposing interaction between spermine and phenylethanolamines at the proton sensor is further supported by the finding that mutations of the NR1 subunit that reduce polyamine potentiation also reduce inhibition by both ifenprodil and protons<sup>37</sup>.

Our results are consistent with these drugs enhancing the inhibitory coupling of the proton sensor to the gating mechanism of the channel by an action on or upstream of the proton sensor. Both protons<sup>7</sup> and ifenprodil<sup>23</sup> reduce the frequency of NMDA channel openings and burst duration, consistent with this proposed mechanism. Thus, phenylethanolamines shift the  $pK_a$  of the proton sensor toward more alkaline levels, a novel mechanism of action for a neuroprotective drug. A given concentration of phenylethanolamine would cause a larger fraction of NMDA receptors to be protonated and consequently inhibited. This mechanism is compatible with earlier findings<sup>24</sup>, which concluded that ifenprodil forces the agonist-bound state of the NMDA receptor into a conformation

with low open probability. Our results indicate that this conformational state is the protonated form of the receptor. Although the inhibitory effect of ifenprodil was predominant, earlier work<sup>24</sup> showed that ifenprodil also causes a small increase in the potency with which glutamate activates NMDA receptors. We found that the potency of NMDA for activating NR1-1a/NR2B receptors was likewise increased more than twofold by 100 nM CP101,606 (Fig. 1d) and about fourfold at pH 6.4 compared with pH 7.6 ( $n = 6$ –8 oocytes at each pH, unpublished observations), suggesting that both actions of the phenylethanolamines (inhibition and increased agonist potency) can be explained by an enhancement of proton effects.

Based on results in this and previous studies<sup>5–7,23,33,34,36–39</sup>, we propose the following model for how phenylethanolamines, spermine, exon 5 and protons control gating of the NMDA receptor (Fig. 7). According to this model, the electronegativity of the proton sensor is a key determinant of the likelihood of the agonist-bound state of the receptor reaching the open channel conformation. Protonation of this sensor could either eliminate a critical protein–protein interaction or allow new interactions to



**Fig. 7.** Schematic diagram of the proposed interaction between phenylethanolamines, spermine, exon 5 and the proton sensor (X<sup>δ-</sup>). See the text for details.

occur. For example, the unprotonated form of this sensor could form a critical hydrogen bond or salt bridge that holds the receptor in a conformation permissive for channel opening. In this case, protonation of the sensor would disrupt the internal interaction, leading to low open probability even in the agonist-bound state. We (Fig. 4c) and others<sup>36,37</sup> have observed that removal by substitution of several acidic residues in NR1 reduces proton sensitivity. This can be understood if the distribution or density of negative charges on the proton-sensing amino acid(s) is influenced by nearby acidic residues. Electron donation by acidic residues (arrow a in Fig. 7) could occur either by induction through the carbon chain or by direct interaction between an electron pair from one or more of the acidic residues and the proton sensor itself.

In the context of this model, phenylethanolamines may stabilize the protonated form of the receptor, leading to the low open probability conformation (arrow b on Fig. 7). Alternately, binding of phenylethanolamines could enhance the ability of one or more identified acidic residues to increase the electronegativity of the proton sensor (arrow c). This possibility is consistent with the observation that removal of negative charge on the acidic residues by mutagenesis weakens ifenprodil potency (Fig. 4c). Exon 5 and/or spermine could act by a shielding mechanism as previously proposed<sup>34</sup> (arrow d). Alternatively, positively-charged exon 5 and/or spermine could reduce the pK<sub>a</sub> of the proton sensor by binding to and neutralizing charges on acidic residues (arrow e). However, for at least one acidic residue, E342, the putative interaction with exon 5 does not seem to occur<sup>33</sup>.

This model points to the proton-sensing amino acid, and its interaction partner(s), as critical elements in the structural basis for gating of NMDA receptor channels. The observed pK<sub>a</sub> is compatible with the proton sensor being either a histidine or cysteine residue, or perhaps a pair of closely spaced acidic residues (see also ref. 40). However, all NR1 cysteines have been removed by substitution without eliminating proton sensitivity<sup>35</sup>, suggesting that one or more histidines, aspartates or glutamates in NR1, or additional residues in NR2, may form the proton sensor. A critical test of the proposed model will require more structural information than is currently available.

Phenylethanolamines represent the latest generation of NMDA receptor antagonists being developed as therapies for stroke and potentially epilepsy. Two of the major challenges to

be overcome in developing a clinically useful drug are to minimize block of NMDA receptors in healthy brain tissue to prevent unacceptable side effects, and to deliver the drug before the stroke, because delivery after experimental stroke is substantially less effective. NMDA receptor antagonists that have been used in early clinical trials for stroke include the competitive antagonists selfotel (CGS 19755) and ACEA 1021, the uncompetitive channel blocker aptiganel (Cerestat, CNS 1102), and the ifenprodil analog eliprodil. The selfotel trial<sup>41</sup> and the eliprodil trial<sup>42</sup> were terminated early because of lack of efficacy at tolerable doses. Eliprodil blocks N, P and Q-type calcium channels at doses that block NMDA receptors<sup>43,44</sup>; this secondary effect could be dose limiting. The new understanding of the mechanism of action of phenylethanolamines on the critical proton sensor suggests an approach to optimize the design of these drugs as neuroprotectants. The interstitial pH is normally around 7.5 but is reduced in ischemic tissue to values as low as 6.5 (ref. 11). The ideal drug would be inactive at physiological pH but active at the lower pH values that occur during ischemia; a promising strategy may therefore be to search for phenylethanolamine compounds that have as high a pharmacological selectivity as CP-101,606 and are inactive at physiological pH, but have a large ifenprodil-like potency increase at ischemic pHs. Such drugs would be expected to be most neuroprotective in brain regions, such as forebrain and hippocampus, that contain high levels of NR2B subunit. Such a context-dependent blocker might be given chronically to individuals at risk for stroke. Moreover, this strategy may hold promise for other proton-sensitive receptors, including GABA<sub>A</sub> and nicotinic acetylcholine receptors (see also refs 45, 46).

#### Methods

**XENOPUS OOCYTE INJECTION AND RECORDING.** All recordings were done in *Xenopus* oocytes injected with mRNA encoding the desired NMDA receptor subunits. The procedure for preparation and injection of oocytes followed that of ref. 47. Cells were injected with 5–15 ng of cRNA encoding either NR1-1a (lacking exon 5) or NR1-1b (containing exon 5) in combination with a 2–3-fold greater amount of NR2A, NR2B, NR2C or NR2D mRNA. Injected oocytes were maintained at 17°C in Barth's solution containing penicillin (10 U/ml) and streptomycin (10 µg/ml) for 2–6 days, after which recordings were made at room temperature from cells continuously perfused in a standard frog Ringer's solution. This solution was composed of 90 mM NaCl, 1.0 mM KCl, 10 or 15 mM HEPES and 1.0 mM BaCl<sub>2</sub>. For some oocytes, 0.1 mM CaCl<sub>2</sub> was added, and the BaCl<sub>2</sub> was reduced to 0.4 mM. This substitution did not significantly alter the inhibitory potency of either ifenprodil or CP-101,606, and so results from oocytes recorded in both solutions were pooled. Where indicated, the ionic strength of the recording solution was reduced by partial removal of NaCl, KCl and HEPES. Recording pipettes were filled with 0.3 M KCl or 3 M CsCl plus 0.4 M EGTA, pH 7.5 to chelate calcium and thereby minimize the activation of calcium-dependent chloride currents. To further minimize these currents, some oocytes were injected with 20 nl of a solution containing 100 mM BAPTA and 10 mM HEPES, pH 7.5, at least 15 min before recording. Near-saturating concentrations of NMDA (100 µM), glutamate (20 µM) and glycine (10 µM) were used to activate the receptor, unless otherwise indicated. CP-101,606 was prepared daily from frozen stock solutions. Ifenprodil was prepared fresh daily from powder. NMDA currents were recorded from oocytes in two-electrode voltage clamp. Currents were typically elicited from a holding potential of -70 mV, except where indicated. Current signals were digitized and recorded using Axotape (version 2.0) or pClamp (version 5.5) software (Axon Instruments, Foster City, California) or custom acquisition software. Current-voltage (I-V) curves were measured using voltage ramps from -100 mV to +50 mV over a period of 1.3 seconds during steady-state responses to either NMDA or NMDA plus CP-101,606. Leak currents, measured by control ramps applied

immediately before and after the test ramps, were averaged and digitally subtracted from currents measured in the presence of NMDA or NMDA plus CP-101,606. At least three ramps were recorded and averaged for each condition in each oocyte. To study the effects of pH, oocytes were perfused with Ringer's solution at the desired pH for 30–60 s or until a stable baseline had been reached before subsequent agonist application.

Application of NMDA and glycine produced a stable, rapidly rising and nondesensitizing current in most oocytes. Oocytes in which the NMDA/glycine current was not stable or in which the baseline holding current at the beginning and end of the experiment drifted by more than 10% were discarded. Inhibition of the NMDA/glycine current by ifenprodil or CP-101,606 was examined by applying up to five different concentrations of the antagonist to each oocyte. Data are expressed as mean  $\pm$  standard error. Unless otherwise noted, the amplitude of the NMDA current at each concentration of antagonist was expressed as a percentage of the control NMDA current in the absence of antagonist.

**IN VITRO NEUROPROTECTION.** Primary cultures of rat cerebral cortex were prepared from Sprague-Dawley rat embryos (E16–E19) essentially as described<sup>48</sup>. Cells were plated into 24-well plates at a density of  $3 \times 10^5$  per well. Plating medium consisted of Neurobasal medium supplemented with L-glutamine (2 mM), penicillin (5 U/ml), streptomycin (10  $\mu$ g/ml) and B-27 supplement. Cells were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub> and fed every four days with plating medium. After 14–22 days in culture, cells were pretreated with ifenprodil (0.003–10  $\mu$ M), CP-101,606 (0.001–10  $\mu$ M) or D-APV (500  $\mu$ M) for 30 minutes in a buffered salt solution at either pH 7.5 (113 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO<sub>4</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 15 mM NaHCO<sub>3</sub> and 10 mM PIPES) or pH 6.5 (identical except 127 mM NaCl and 1.5 mM NaHCO<sub>3</sub>). Excitotoxicity was induced by treating cultures with saturating concentrations of NMDA (0.5–1 mM) and glycine (10–100  $\mu$ M) for 10 minutes in the presence of ifenprodil or CP-101,606, after which plates were rinsed twice with fresh medium containing D-APV (300–500  $\mu$ M) and CNQX (10  $\mu$ M) and returned to the incubator. D-APV and CNQX were added to limit the period of excitotoxicity to the 10-min exposure. After 24 h, excitotoxic damage was assessed by measuring the amount of lactate dehydrogenase (LDH) released into the culture medium (Tox-7 kit; Sigma Chemical Co, St. Louis, Missouri). Released LDH was expressed as the fraction of total LDH present in each well. NMDA-evoked excitotoxicity was defined as the amount of LDH released by NMDA and glycine in the absence of phenylethanolamine, minus the amount of LDH released from cultures pretreated with D-APV. Cultures in which the NMDA-evoked excitotoxic cell death was less than 10% were discarded. The neuroprotective effect of either ifenprodil or CP-101,606 in each experiment was expressed as the percentage of the total NMDA-mediated cell death that occurred in the presence of the phenylethanolamine.

**MATERIALS.** CP-101,606 [(1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol] was a gift from Pfizer (Groton, Connecticut). The NR1-1a mutants E339Q, E181Q, E342Q, D669N, and D669E were donated by Keith Williams; NR1(N616Q) was provided by K. Moriyoshi. NMDA, D-APV and CNQX were purchased from Tocris Cookson (St. Louis, Missouri). Ifenprodil, glycine and all other reagents for electrophysiology were purchased from Sigma Chemical Company (St. Louis, Missouri). Neurobasal media, B-27 supplement, L-glutamine and other cell culture reagents were purchased from Gibco BRL (Grand Island, New York). Molecular biology reagents and restriction enzymes were purchased from Promega (Madison, Wisconsin), Pharmacia (Piscataway, New Jersey) and Boehringer Mannheim (Indianapolis, Indiana).

**DATA ANALYSIS.** The amplitude of currents recorded from oocytes was measured using Origin (Microcal Software, Inc, Northampton, Massachusetts) or custom software. Concentration–response curves represent a least-squares fit of each data set to a sigmoidal (logistic) curve (GraphPad Prism, GraphPad Software, San Diego, California). The IC<sub>50</sub>, IC<sub>25</sub>, Hill slope and maximal blockade produced by the antagonist were calculated from this curve. Means, standard errors and 95% confidence intervals (95% c.i.) were determined by the fitting algorithm. Statistical significance was determined using one way analysis of variance (ANOVA) with post-hoc Bonferroni tests or the Student's *t*-test, with  $\alpha < 0.05$ .

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