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The novel NMDA receptor antagonist, 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid, is a gating modifier in cultured mouse cortical neurons

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Abstract

Neu2000 [NEU, 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid], a derivative of sulfasalazine, attenuates NMDA-induced neuronal toxicity. Here we investigated the effects of NEU on the NMDA receptor (NMDAR) using whole-cell patch clamp technique to determine the molecular mechanisms underlying its neuroprotective role. NEU reversibly suppressed NMDA responses in an uncompetitive manner with fast binding kinetics. Its inhibition of NMDAR activity depended on both the concentration and the use of agonist but not on the membrane potential. NEU accelerated NMDA desensitization without affecting the binding affinity of NMDAR for its agonists and stabilized the closed state of NMDAR. Therefore, NEU should effectively alleviate disorders that are a result of glutamate excitoxicity with fewer side effects because it is a low-affinity gating modifier that antagonizes NMDAR in an uncompetitive manner. Moreover, in the presence of ifenprodil (an NR2B antagonist) but not NVP-AAM077 [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl]-phosphonic acid, an NR2A antagonist], the extent of NEU block was decreased, suggesting that NEU is an NR2B-specific antagonist.

Keywords: aspirin, calcium, electrophysiology, glutamate, stroke.

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Excitotoxic neuronal death because of the overactivation of the NMDA receptor plays a key role in the etiologies of hypoxic-ischemic brain injury, trauma, and several degenerative neurological diseases (Rothman and Olney 1986). Naturally, this has elicited considerable interest in the therapeutic potential of drugs that target NMDARs. Disappointingly, to date, numerous NMDAR antagonists have failed to show beneficial effects in human clinical trials of disorders including stroke (Kemp and McKernan 2002), and the theory of glutamate excitotoxicity as a foundation for the development of neuroprotective therapies has been even questioned (Ikonomidou and Turski 2002).

The failure of clinical trials for NMDA antagonists results from their marked side effects and toxicity (Kemp and McKernan 2002; Muir 2006). High-affinity non-competitive as well as competitive antagonists exhibit severe psychomimetic side effects (Muir 2006). Open-channel blockers with an uncompetitive mode of action accumulate in the pore region of NMDAR, which makes them not useful for clinical application. However, memantine, an uncompetitive open-channel blocker with relatively low affinity appears not to exhibit significant side effects at doses used in clinical practice (Robinson and Keating 2006). Amantadine is used to treat Parkinson's disease (Blanchet *et al.* 2003), and is a low-affinity NMDA receptor channel blocker (Parsons *et al.* 1995). The precise mechanism underlying the beneficial effect of low-affinity antagonists remains uncertain; however, their relatively fast off-rate has been proposed to give them fewer side effects (Kemp and McKernan 2002; Lipton 2004). Although it has been shown to be ineffective in a clinical trial treating traumatic injury, the NR2B-selective antagonist, CP-101606, does not have adverse effects (Merchant *et al.* 1999), raising the possibility that the subtype selectivity of NMDAR antagonists is important in reducing their side effects and toxicity. Therefore, NMDAR subtype-specific antagonists with low affinity may exhibit fewer side-effects and toxicity.

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Abbreviations used: DIV, days *in vitro*; NEU, 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid; Ca^{2+}_{ex}, extracellular Ca^{2+}; s-s/P, steady-state/P; NVP-AAM077, [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl]-phosphonic acid.

In addition to issues with side-effects and toxicity, problems with NMDAR antagonists have been proposed to originate from their narrow post-insult therapeutic time windows (Ikonomidou and Turski 2002). Reperfusion injury triggered by cytotoxic-free radicals is one of the most important factors determining the post-insult therapeutic time window. Sulfasalazine prevents NMDA-induced neuronal death and attenuates free radical injury (Ryu *et al.* 2003). Considerable amounts of evidence show that this anti-inflammatory agent, recently identified as a non-competitive NMDAR antagonist (Noh *et al.* 2006), plays a neuroprotective role in acute and chronic neurodegenerative diseases (Rich *et al.* 1995; Barneoud and Curet 1999). Based upon the pharmacological actions of sulfasalazine, the novel neuroprotectant 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid was designed and named 'Neu2000/NEU'. NEU is much more potent than sulfasalazine in protecting neurons against NMDAR-mediated excitotoxicity and free radical injury (Gwag *et al.* 2007). NEU dramatically reduces glutamate toxicity and prevents ischemic cell death with a fairly long therapeutic window. A thorough understanding of the interaction between NMDAR and NEU will permit us to develop a more safe and effective NMDA antagonist. In this study, therefore, using whole-cell patch recording, we investigated the molecular profile of NEU in mouse cortical cell cultures to determine the mechanisms by which NEU inhibits NMDA response.

Materials and methods

Preparation of cortical cell cultures

Cortical cell cultures were prepared from 15-day-old ICR mouse embryos. Fetuses were removed from the uterus and decapitated with fine forceps. Cerebral cortices were harvested and were gently triturated with fire-polished Pasteur pipettes. Cortical cells (approximately 10^6 cells/dish) were plated onto 35-mm plastic Petri dishes pre-coated with poly-D-lysine (100 μg/mL, Sigma, St Louis, MO, USA) and laminin (4 μg/mL, Sigma). The plating medium was Eagle's minimum essential medium (Invitrogen Co., Carlsbad, CA, USA) supplemented with 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose. Experiments were performed at 20 ± 2°C on 11–19 days *in vitro* (DIV) after plating.

Electrophysiological recordings

Whole-cell currents were recorded using an Axopatch 200A amplifier with a Digidata-1322 A/D converter and the software pCLAMP9 (Molecular Devices, Sunnyvale, CA, USA). Neuronal currents were filtered at 5 kHz and sampled at 0.2–5 kHz. The resistance of recording pipettes of borosilicate capillaries (King Precision Glass, Inc., Claremont, CA, USA) was typically 2–3 MΩ. Pipette seal resistances were > 10 GΩ and the junction potential was 5 mV. Recordings were taken at a holding potential of −60 mV unless otherwise specified. Data graphing and dose–response analyses were performed with the GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean ± SEM. Student's *t*-test was employed to determine the statistical significance of differences between two groups; ANOVA with Newman–Keuls *post hoc* test was used for multiple comparison. The best-fit curve for concentration–response relations was constructed by fitting the data to the following Hill's equation:

$$\% \text{ of control} = [(\max - \min)/\{1 + (x/z)^n\}] + \min$$

where max = maximum response, min = minimum response, x = concentrations of agonist or antagonist, z = the concentration of agonist or antagonist at 50% of the maximum response, and n is Hill's coefficient.

Solutions and chemicals

The electrode pipettes were filled with an internal solution containing (in mM) 135 CsCl, 10 HEPES, 1.2 $MgCl_2$, 4 ATP-Na_2, 0.5 $CaCl_2$, and 11 EGTA (pH adjusted to 7.3 with CsOH). The external recording solution comprised of (in mM) 140 NaCl, 2 KCl, 2 $CaCl_2$, 10 HEPES, 10 D-glucose, and 0.01 glycine (pH adjusted to 7.4 with NaOH). These internal- and external-solutions were used unless otherwise stated. NEU was prepared as a stock solution (100 mM) in dimethyl sulfoxide. [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077/NVP) was prepared as a stock solution in 0.1 N NaOH.

External recording solutions were applied using a gravity-driven perfusion system with a linear array of barrels. High concentrations of NMDA (1–5 mM) were perfused onto a cell using Picospritzer (General Valve Co., Fairfield, NJ, USA), where required.

Results

Agonist-dependent antagonism of NEU

As the first step to lighten the action mechanism of NEU as an NMDAR antagonist, we assessed both the degree of blockade and kinetics of NEU action on steady-state NMDA responses at two different concentrations (30 and 300 μM). NEU blocked the response of 300 μM NMDA more effectively. The mean value of the steady-state NMDA currents blocked by NEU was significantly greater in 300 μM (66%) than 30 μM (54%) NMDA (Fig. 1a), indicating that NEU blockade depends on the agonist concentration. Additionally, the mean on-rate time constant (τ_{on}) of block decreased significantly as the NMDA concentration increased (Fig. 1b), suggesting that higher the agonist activity, the more effectively NEU acts on the NMDAR. No significant changes in the mean off-rate time constant of block (τ_{off}), however, were observed, indicating that recovery from block was not accelerated by the agonist. From the on- and off-rate time constants of block, we calculated an equilibrium dissociation constant (K_D) for NEU in 300 μM NMDA at varying NEU concentrations according to a simple bimolecular reaction scheme (Hille 2001). The plot of $1/\tau_{on}$ versus NEU concentration was linear (Fig. 1c), indicating

that higher concentrations of NEU led to the inhibition to proceed more quickly. From the slope of this plot, the blocking rate constant (k_{on}) was determined to be 15.1×10^3/M·s and from the zero *y*-intercept, the unblocking rate constant (k_{off}) was estimated to be 1.1/s, yielding a K_D = 74.8 μM from the equation $K_D = k_{off}/k_{on}$. The inverse of τ_{off} did not correlate with antagonist concentration. Such an increase of the blocking, but not unblocking, rate of NEU reflects an apparent increase in the affinity for NEU.

To further investigate whether the binding affinity of NEU for the NMDAR is altered at different NMDA concentrations, NEU inhibition curves were obtained with 30 and 300 μM NMDA (Fig. 1d). The IC_{50} values of the inhibition curves were 120.2 ± 6.1 μM for 30 μM NMDA (n = 5) and 35.4 ± 6.1 μM for 300 μM NMDA (n = 8), representing a more than threefold shift. This strongly indicates that NEU antagonism is dependent on NMDA concentration. The agonist concentration dependency of NEU block was further supported by the dose–response relation of NMDA. NEU depressed the maximum NMDA response as well as the EC50 value in a concentration-dependent manner (Fig. 1e), clearly showing an uncompetitive antagonism of NEU.

Use- but not voltage-dependent NMDA reduction by NEU

The uncompetitive antagonism of NEU with clear agonist dependency suggests that NEU acts as an open-channel blocker like memantine and amantadine, which inhibit NMDA responses in a voltage- and use-dependent manner (Dingledine *et al.* 1999). To examine this possibility, we first compared the degree of NEU block at two different holding potentials (−60 and +50 mV). NEU reduced the steady-state NMDA currents to 34.0 ± 3.0% and 30.0 ± 5.0% of control values at −60 and +50 mV, respectively (n = 10; $p > 0.05$; Fig. 2a, left), indicating that the NEU-induced suppression is not significantly dependent on membrane potential. We expanded our investigation to a voltage range of −110 to +70 mV. No significant alterations in the NEU block were observed over the applied voltages (n = 4, $p > 0.05$), showing a voltage-independent reduction of about 70–80% (Fig. 2a, right). In addition, the reversal potential of NMDA response was not affected by NEU.

We additionally analyzed whether NEU reduced NMDA responses in a use-dependent manner (Fig. 2b, upper). Once a stable control response was established, NMDA was repetitively applied in the presence of NEU. No progressive decline in successive NMDA response was observed in the presence of NEU. Notably, the unblocking rate of NEU was really fast (k_{off} = 1.1/s). To avoid experimental errors possibly because of the limited time resolution of our manually driven solution exchange system, we evoked a transient 5 mM NMDA response repetitively by a pressure ejector and examined the effect of NEU on it. The first NMDA spike after NEU exposure (* in Fig. 2b, lower) became significantly larger than the following NMDA responses under NEU (n = 5), showing the use-dependent nature of NEU block.

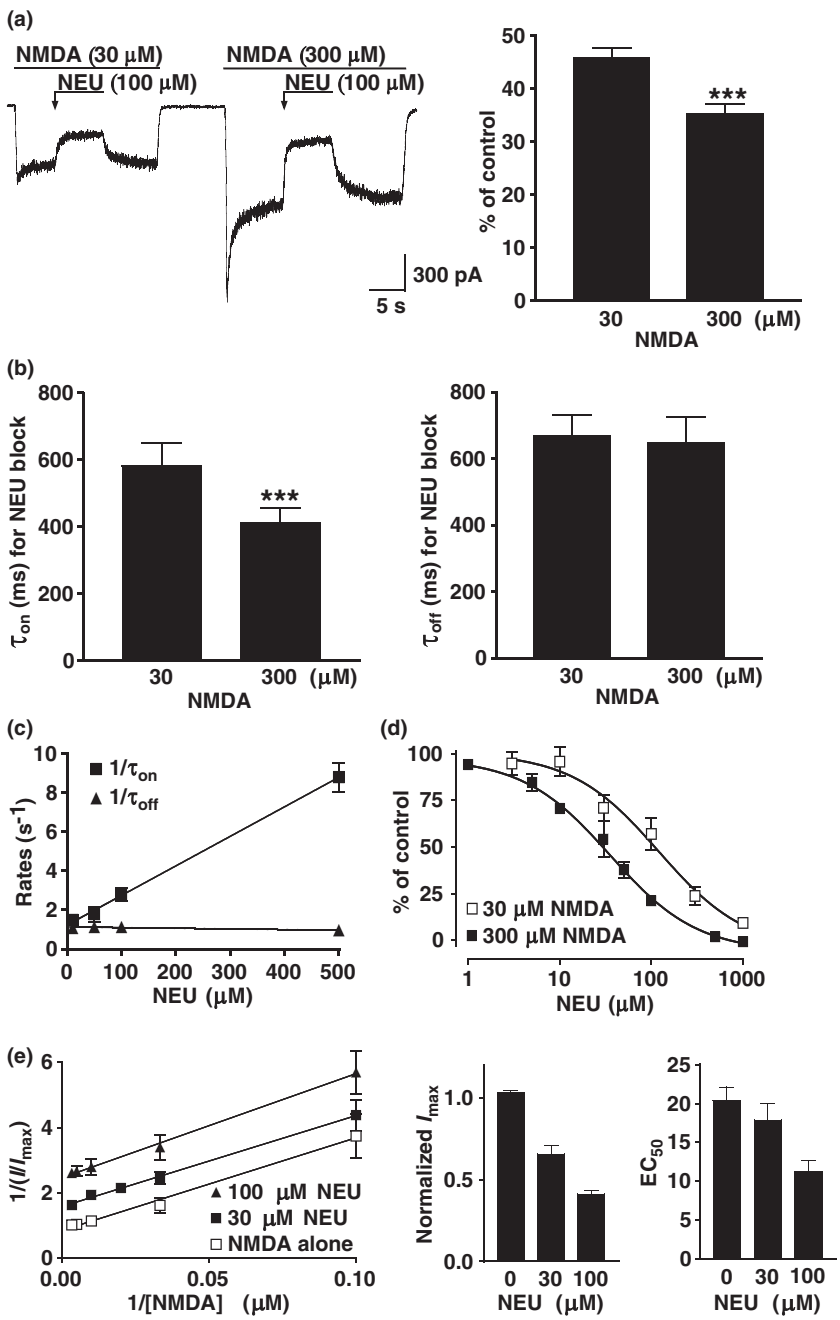
Acceleration of NMDAR desensitization and stabilization of the NMDAR closed states by NEU

Use-dependent blockade does not necessarily mean blockade of the open pore or binding preferentially to the open state (Orser *et al.* 1997). The agonist- and use-dependency of NEU may imply a preferential binding of NEU to an agonist-associated closed state. It was noteworthy that the K_D (74.8 μM) based on rate constants was larger than the IC_{50} (35.4 μM) of the inhibition curves, which implies that NEU inhibits total NMDAR-related responses more effectively than if it only affected a transition from the open to the closed state. Three possible factors can be considered to explain the divergence of K_D and IC_{50} of NEU: a reduction of agonist binding affinity, an enhancement of desensitization, or a stabilization of the closed state (Blanpied *et al.* 2005).

To determine the mechanisms by which NEU suppresses NMDA responses, we examined NMDA binding kinetics in the presence of NEU. NEU apparently increased the time constant for NMDA activation (τ_{act}) from 34.4 to 49.1 ms and for NMDA deactivation (τ_{deact}) from 197.3 to 337.7 ms (Fig. 3a, left). However, statistics revealed that the changes in NMDA binding kinetics by NEU were not significant (Fig. 3b). Notably, NMDA-evoked currents were reduced to 65% at steady state, but to only 39% at the peak in the continual presence of NEU (n = 13; Fig. 3a, right), suggesting that NMDARs are desensitized in the presence of NEU as the steady-state/peak (s-s/P) ratio for the block represents whether the blocker inhibits channel desensitization (Sobolevsky *et al.* 1999). Time constants for desensitization (τ_{desen}) of NMDA-elicited responses were significantly reduced in the presence of NEU (Fig. 3b, right). These results imply that NEU at least partially attenuates the NMDA response by accelerating NMDAR desensitization without affecting either association or dissociation of NMDA.

The reduction of NMDAR response in the presence of agonist can occur by three different mechanisms: glycine-dependent (Mayer *et al.* 1989), glycine-independent (Sather *et al.* 1990), and Ca^{2+}-dependent desensitization (Legendre *et al.* 1993). It seems unlikely that glycine-dependent desensitization contributes to the acceleration of desensitization by NEU as seen in Fig. 3a, as a saturating concentration of glycine (10 μM) was used in this experiment. Ca^{2+}-dependent desensitization results from intracellular Ca^{2+} transients following the opening of either NMDA or voltage-gated Ca^{2+} channels (Legendre *et al.* 1993). Therefore, we compared the degree of NEU block at two different levels of extracellular Ca^{2+} (Ca^{2+}_{ex}). Under saturating NMDA and glycine conditions, both peak and steady-state NMDA

currents became larger in 0.2 mM Ca^{2+}_{ex} than in 2 mM Ca^{2+}_{ex} (Fig. 4a). The value of s-s/P for the NMDA response was greater in 0.2 mM Ca^{2+}_{ex} (s-s/P = 0.57) than in 2 mM Ca^{2+}_{ex} (s-s/P = 0.47), showing that the lower the Ca^{2+}_{ex}, the less Ca^{2+}-dependent desensitization occurs. At 2 mM Ca^{2+}_{ex}, NEU co-applied with NMDA decreased the value of s-s/P by 28% and τ_{desen} by 30%, confirming the accelerating effect of NEU on NMDAR desensitization shown in Fig. 3. At 0.2 mM Ca^{2+}_{ex}, NEU also decreased both the s-s/P value and τ_{desen} by 40% and 26%, respectively. Interestingly, NEU appeared to increase the peak NMDA amplitude at 0.2 mM Ca^{2+}_{ex}. The decrease of Ca^{2+}_{ex} from 2 to 0.2 mM decreased the degree of NEU block of steady-state NMDA currents significantly (Fig. 4b), suggesting that a Ca^{2+}-dependent inactivation responsible for the slow component of NMDAR desensitization (Krupp *et al.* 1996) contributes to NEU-enhanced desensitization. However, the reduction of τ_{desen} by NEU was not significantly altered by lowering Ca^{2+}_{ex}. The reduction of the slow portion of NMDAR desensitization by NEU would likely be a reflection of glycine-independent but



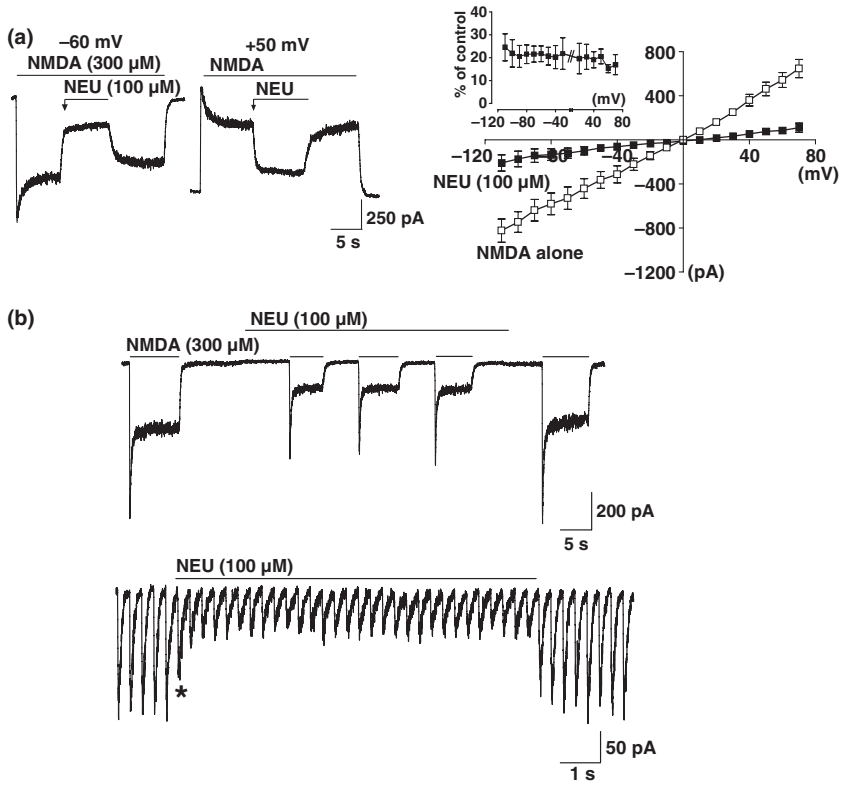


Fig. 2 Voltage-independent but use-dependent characters of NEU activity. (a) NEU (100 μM) was applied to NMDA (300 μM) currents elicited from a cortical neuron held at either −60 or +50 mV (left). No significant differences were observed in NEU block of NMDA responses between −60 and +50 mV ($n = 10$; $p > 0.05$). I–V NMDA (300 μM) responses were examined in 10 mV intervals over a voltage range between −110 and +70 mV in the absence (right, NMDA alone; □) and presence of NEU (100 μM NEU; ■). The inset shows the average changes in NEU block over the voltage tested was not statistically significant (ANOVA, $p > 0.05$; $n = 4$). (b) Following a control response to 300 μM NMDA, NEU (100 μM) was applied to the bath. In the presence of NEU, repeated NMDA-evoked currents exhibited similar responses; after wash-out of NEU, the NMDA response recovered completely (upper trace). Similar response profiles were observed from all neurons tested ($n = 5$). Five mM of NMDA was applied consecutively (every 400 ms) to neurons held at −60 mV, using Picospritzer (68.9 kPa, 4 ms), to elicit transient NMDA responses, and after the stabilization of the transient NMDA responses were obtained, NEU (100 μM) was added (lower trace). The first NMDA spike (*) or only a couple of initial spikes were partially antagonized by NEU ($n = 5$).

Fig. 1 Agonist-dependent nature of NEU action. (a) Current trace changes were induced by NEU applied to cortical neurons during pulses of two different concentrations of NMDA (left). NEU (100 μM) significantly antagonized NMDA responses in an agonist concentration-dependent manner; the mean normalized values were 45.8 ± 2.0% for 30 μM NMDA and 35.3 ± 1.8% for 300 μM NMDA, respectively (***$p < 0.001$, $n = 11$) (right). (b) On-rate (left, τ_{on}) and off-rate (right, τ_{off}) time constants for NEU-induced suppression of 30 and 300 μM NMDA responses were determined by fitting the digitized data (a) to a single exponential function. The τ_{on} for NEU block was significantly smaller in the presence of the higher concentration of NMDA (580.4 ± 68.8 ms for 30 μM NMDA; 411.5 ± 44.2 ms for 300 μM NMDA; ***$p < 0.001$, $n = 11$), whereas the τ_{off} for NEU block was independent of NMDA concentration (668.3 ± 64.5 ms for 30 μM NMDA; 648.5 ± 75.8 ms for 300 μM NMDA; $p > 0.05$, $n = 11$). (c) Kinetic analysis of the action of NEU on NMDAR binding sites. The reciprocal values of the on-(■) and off-(▲) rate time constants were plotted as a function of NEU concentration by a simple bimolecular reaction. Lines were fitted by linear regression with each point representing the mean ± SEM ($n = 6$). (d) Inhibition curves for the NEU-induced antagonism of steady-state responses to NMDA at either 30 or 300 μM NMDA. The amplitude of the current response in the presence of NEU is expressed as a percentage of the control responses (% of control). The IC_{50} value and Hill's coefficient were 120.2 ± 6.1 μM and 0.9 ± 0.4 ($n = 5$) for 30 μM NMDA (□) and 35.4 ± 6.1 μM and 0.9 ± 0.2 ($n = 8$) for 300 μM NMDA (■), respectively. (e) Steady-state NMDA-evoked currents (I) were normalized to maximal NMDA responses (I_{max}) to obtain Lineweaver-Burk plots for the NMDA dose relation in the absence (NMDA alone; □) and the presence of NEU (30 μM, ■; 100 μM, ▲) (left). NEU significantly decreased the normalized maximum NMDA responses (middle, NMDA alone: 1.03 ± 0.02, $n = 11$; 30 μM NEU: 0.66 ± 0.05, $n = 8$; 100 μM NEU: 0.41 ± 0.02, $n = 5$; ANOVA, $p < 0.0001$) and EC_{50} values (right, NMDA alone: 20.34 ± 1.77 μM, $n = 11$; 30 μM NEU: 17.87 ± 2.11, $n = 6$; 100 μM NEU: 11.25 ± 1.38, $n = 5$; ANOVA, $p < 0.05$).

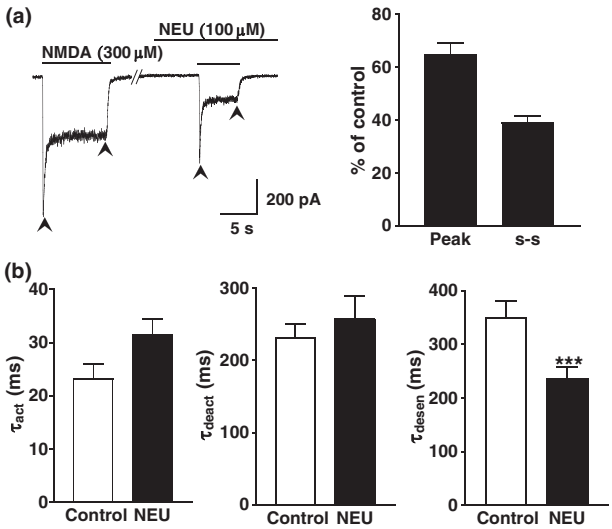


Fig. 3 Effect of NEU on NMDAR kinetics. (a) NMDA pulses were generated on a cortical neuron (DIV 12) in the continual presence of 100 μM NEU (left). NEU decreased the peak NMDA amplitude (Peak) by 36% and the steady-state current by 58%. The right panel shows the mean percent values of NMDA currents in the presence of NEU relative to controls (n = 13; Peak, 64.6 ± 4.4%; s-s, 39.0 ± 2.7%). (b) Bar graphs show that NEU affected neither the association nor dissociation kinetics of the NMDA responses. The time constants for association (τ_{act}) in the control samples (Control) and NEU-treated cells (NEU) determined by fitting the rising currents (from the start of the current to the first arrowhead indicated) to an exponential power function (power factor = 2) were 23.1 ± 3.0 and 31.5 ± 2.9 ms, respectively (n = 8, $p > 0.05$; left). The time constants for dissociation (τ_{deact}) measured by fitting the declining current (from the second arrowhead to the end of the current) to a single exponential function were 230.4 ± 20.4 and 256.9 ± 32.7 ms, respectively (n = 10, $p > 0.1$; middle), while those for desensitization (τ_{desen}) determined by fitting the decay currents (between two arrowheads) to a single exponential function were 350.1 ± 30.3 and 236.2 ± 21.0 ms, respectively (n = 13; ***$p < 0.001$; right).

Ca^{2+}-dependent desensitization not resulting from Ca^{2+}-dependent inactivation itself. It is worth noting that NMDA peak currents were reduced by NEU at 2 mM Ca^{2+} while remaining intact or increasing at 0.2 mM Ca^{2+}. This dramatic effect of NEU on the fast peak current strongly suggests that glycine-independent/Ca^{2+}-dependent desensitization in charge of the fast decay of NMDA currents (Legendre *et al.* 1993) is one of the main targets of NEU action.

Next, to determine the effect of NEU on the closed state of NMDARs, we minimized desensitization with low-Ca^{2+} (0.2 mM) + high-glycine (10–20 μM) extracellular medium and intracellular inclusion of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′*,N′-tetraacetic acid (BAPTA). The effect of NEU on the closed channel is shown in Fig. 4c, where NMDA current was elicited after a 35-s application of 100 μM NEU. The NEU was washed off for ~100 ms before reapplication of NMDA in order to remove any inhibitory effect of residual antagonists after wash-off. The transient NMDA responses preceded by NEU but not steady-state currents were significantly reduced by 11.0 ± 2.2% (n = 9, $p < 0.001$), indicating that NEU inhibited the response by stabilizing the closed state of NMDARs. The on-rate of NMDA response appeared to become slower after pre-treatment of NEU, however, this was not statistically significant (n = 9, $p > 0.05$).

Specificity of NEU action on NMDAR subtypes

As mRNAs encoding NR2A and NR2B are significantly expressed in murine cortical cultures (Mizuta *et al.* 1998), mouse cultured cortical neurons used in this study are likely to have both subtypes of NMDA receptor. A subtype-specific antagonist would be then expected to decrease the degree of NEU block if NEU antagonized this subtype selectively.

To address whether NEU selectively blocked NR2B receptors, we compared the degree of NEU block in the presence of either NR2B or NR2A receptor antagonists. Ifenprodil inhibits NMDA currents of NR1/NR2B receptors with high affinity (IC_{50} = 0.34 μM) and of NR1/NR2A receptors with low affinity (IC_{50} = 146 μM) (Williams 1993). Less than 100 nM NVP selectively blocks NR2A receptors without affecting NR2B response (Auberson *et al.* 2002). Therefore, 50 nM of NVP was applied to obtain the best possible selectivity between mouse NR2A and NR2B subunits. Ifenprodil (0.5 μM) inhibited 57% of NMDA steady-state currents from cortical neurons at DIV 17 while NVP (50 nM) inhibited only 13% of cellular NMDA currents at DIV 19 (Fig. 5a). The mean value of the steady-state NMDA currents (53%) blocked by ifenprodil was significantly (n = 5, $p < 0.01$) greater than that (24%) blocked by NVP (Fig. 5b, left), suggesting that NR2B receptors are dominantly expressed in mouse cultured cortical neuron at DIV 17–19. It is noteworthy that, at mouse cortical cultures, the expression of NR2A mRNA is significant even at DIV 1 and is quite comparable with that of NR2B mRNA at DIV 18; however, western blot detects only NR2B receptor at DIV 11 (Mizuta *et al.* 1998), implying that the expression of NR2A receptor molecule is lower than that of NR2B one. The degree of NEU block on steady-state current appeared to be smaller in the presence of ifenprodil (49%) compared to control (64%). On the other hand, no prominent change in the degree of NEU block on steady-state current was observed with 50 nM NVP (Fig. 5a). Ifenprodil (0.5 μM) significantly decreased the degree of NEU block (n = 5, $p < 0.0001$). NEU (100 μM) alone decreased NMDA currents by 65%, whereas NMDA currents remaining in the presence of ifenprodil were reduced by 50% (Fig. 5b, middle). The reducing effect of ifenprodil on NEU block was concentration-dependent on ifenprodil; NEU only decreased 19% of NMDA currents in the presence of

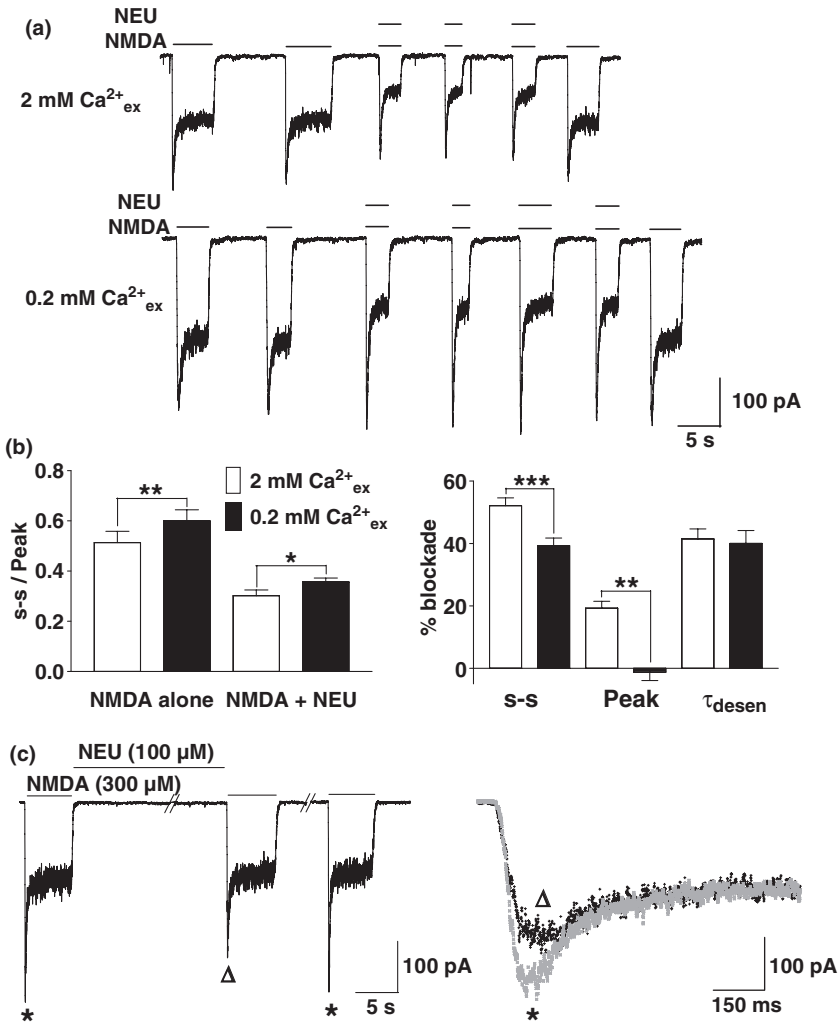


Fig. 4 Effect of NEU on NMDAR desensitization and closed states. (a) The raw traces of NMDA (300 μM) currents elicited from cultured cortical neurons (DIV 19) either in 2 or 0.2 mM Ca^{2+} solutions with 20 μM glycine. The lowering of Ca^{2+} in external solution increased the ratio of steady state to Peak (s-s/P) NMDA response from 0.47 to 0.57. NEU (100 μM) decreased the values of s-s/P from 0.47 to 0.34 and from 0.57 to 0.34, respectively, in 2 and 0.2 mM Ca^{2+}_{ex}. Time constants for desensitization (τ_{desen}) were also decreased by NEU from 327 to 228 ms and from 323 to 238 ms in 2 and 0.2 mM Ca^{2+}, respectively. (b) Bar graphs display the effect of external Ca^{2+} on the mean values of s-s/Peak and of percent blockade. The lowering of external Ca^{2+} from 2 to 0.2 mM significantly increased the value of s-s/P of NMDA responses from 0.51 ± 0.04 to 0.60 ± 0.04 (NMDA alone; $n = 6$, $^{**}p < 0.01$) and also increased the responses in the presence of NEU (NMDA + NEU: $n = 6$, $^{*}p < 0.05$; 2 mM Ca^{2+}_{ex}: 0.30 ± 0.02; 0.2 mM Ca^{2+}_{ex}: 0.36 ± 0.02). The percent values of NEU block on both s-s and Peak were also affected significantly by the lowering external Ca^{2+} from 2 to 0.2 mM, from 52.1 ± 2.5 to 39.3 ± 2.5%, and from 19.3 ± 2.2 to −1.2 ± 2.7%, respectively ($n = 6$; $^{***}p < 0.001$, $^{**}p < 0.01$). No significant percent reductions in τ_{desen} were observed ($n = 6$; 41.5 ± 3.2% in 2 mM Ca^{2+}_{ex}; 40.0 ± 4.1% in 0.2 mM Ca^{2+}_{ex}). (c) The application of NMDA was preceded by NEU exposure for 35 s (left trace). The pre-treatment of NEU (100 μM) prominently reduced the peak (Δ) of the following NMDA current. The right traces show the superimposed control NMDA currents (*, gray; two averaged currents) and currents after NEU treatment (Δ, black) in the left traces. For this experiment, the internal recording solution contained (in mM) 120 cesium methane sulfonate, 10 CsCl, 10 HEPES, 10 BAPTA, 4 ATP-Na_2, pH 7.36. The 0.2 mM Ca^{2+} external solution was supplemented with either 10 or 20 μM glycine.

10 μM ifenprodil. No significant changes in the degree of NEU block were observed in the presence of NVP, suggesting that NEU selectively blocks NR2B receptor (Fig. 5b, right).

Discussion

The major finding in this study is that NEU acts as a rapid NR2B-specific NMDAR gating modifier with uncompetitive

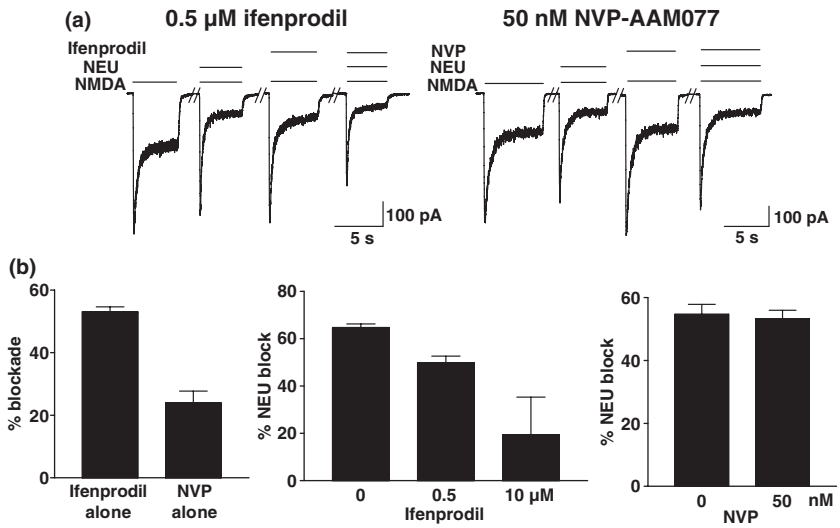


Fig. 5 Selectivity of NEU antagonism for the NR2B receptor. (a) NEU (100 μM) and/or ifenprodil (0.5 μM) were co-applied with NMDA (300 μM) to a cortical neuron (DIV 17) held at −60 mV (left). Fifty seven percent of the steady-state NMDA current was reduced by 0.5 μM ifenprodil. NVP (50 nM; right) also decreased only 13% of the steady-state current elicited from a cortical neuron at DIV 19. Notably, neither ifenprodil nor NVP affected the peak NMDA currents. All of these experiments were carried out under 20 μM glycine. (b) Bar graphs show a summary of the data in the above figure. Ifenprodil (0.5 μM) and NVP (50 nM) blocked 53.0 ± 1.6% ($n = 5$) and 23.9 ± 3.8% ($n = 6$) of the steady-state NMDA (300 μM) currents, respectively (left). In the presence of ifenprodil, the degree of NEU block on the steady-state NMDA current was decreased from 64.7 ± 1.6% (NEU alone, $n = 8$) to 49.8 ± 2.8% (0.5 μM ifenprodil, $n = 5$) and 19.4 ± 15.9% (10 μM ifenprodil, $n = 3$) significantly (ANOVA, $p < 0.0005$; middle) whereas it was not significantly decreased in the presence of NVP (50 nM) ($n = 6$; right).

antagonism. NEU antagonizes NMDA responses through an acceleration of receptor desensitization as well as a stabilization of the closed state of the receptor. This combination of mechanisms underlying NEU inhibition may favor the potential application of NEU in the prevention of neurological diseases.

Molecular mechanisms underlying the NEU blockade of NMDA signals

All effects of NEU on NMDARs are rapid and reversible. The fast action of NEU and rapid cellular recovery rules out possible intracellular action. The fast on-rate of NEU block increased as the concentration of NMDA increased and both the inhibition curves and dose–response relationships demonstrated that NEU block depended on agonist concentration (Fig. 1). These agonist-dependent properties of NEU inhibition suggest that NEU is an open-channel blocker with uncompetitive antagonism. However, several lines of counter-evidence are provided. First, NEU did not display the typical characteristics of a channel pore blocker such as a voltage-dependent block (Fig. 2a). Second, the lack of effect on reversal potential of the I–V curves indicated that NEU did not bind or modify a selectivity filter located at the pore of the channel. Third, neither NMDA-'hooked' currents after co-application with NEU (Figs. 4a and 5a) nor NMDA-'tail' currents in the continuous presence of NEU (Fig. 3a) were observed. In principal, when fast open-channel blockers dissociate from the channel, hooked currents are generated at the end of the co-application of agonist and antagonist or tail currents in the continuous presence of antagonist (Sobolevsky *et al.* 1999). Also, the overshoot that appears when a blocker is removed in the continual presence of agonist (Sobolevsky *et al.* 1999) was not elicited by NEU (Figs. 1a and 2a). Combined with the lack of data indicating a voltage-dependent nature, these phenomena therefore clearly indicate that NEU does not block the open NMDAR channel.

Open-channel blockers are generally classified as uncompetitive antagonists in that they act only on the activated receptor. However, it is not necessary to say that uncompetitive antagonists with agonist dependency must be open-channel blockers. Ifenprodil is not an open-channel blocker but a gating modifier (Legendre and Westbrook 1991) and antagonizes NMDARs in an activity-dependent, uncompetitive manner (Kew *et al.* 1996). More interestingly, it was recently argued (Gilling *et al.* 2007) that the equilibrium blocking potency of memantine, a well-known uncompetitive NMDAR open-channel blocker, depends on agonist concentration. All these arguments imply that an uncompetitive antagonist with agonist dependency is not necessarily an open-channel blocker. Therefore, we have assumed that the agonist- and use-dependency of NEU results from the preferential binding of NEU to an agonist-associated closed state. We have noted that the K_D value (74.8 μM) estimated

from the kinetic parameters (Fig. 1c) is twice that determined from the equilibrium inhibition analysis (IC_{50} = 35.4 μM) (Fig. 1d). We regard the *macroscopic* K_D value as a reflection of gross estimates of *microscopic* rate constants under the assumption that antagonist binding and unbinding are rate-limiting steps because the kinetics of NMDA action on NMDAR is over 20 times faster than that of NEU. NEU would be likely to inhibit NMDA responses more than by simply affecting a transition from the open to the closed state.

One possible explanation for the divergence of K_D and IC_{50} of NEU would be a reduction of NMDA affinity resulting in a competitive antagonism. This should not be the case for NEU. Indeed, NEU did not affect both on- and off-rate of NMDA significantly (Fig. 3b). It is more likely that NEU acts on the NMDAR to reduce the coupling between NMDA binding and channel gating or to stabilize the desensitized NMDAR state. The value of s-s/P at the co-application of NEU with NMDA was significantly smaller than that in the continual presence of NEU (Figs. 3 and 4). It suggests that NEU pre-exposed to the closed states of NMDAR affects the NMDAR activation. NEU pre-treatment reduced the initial peak response of NMDAR significantly (Fig. 4c), showing that a stabilization of the channel closed state by NEU is responsible for the reduction in channel gating. As IC_{50} decrease with NMDA concentration, we also expect that NEU preferentially stabilizes the desensitized state of NMDAR according to the channel block models previously proposed (Orser *et al.* 1997; Sobolevsky *et al.* 1999). Figure 4 clearly shows that enhancement of desensitization by NEU is one of the main mechanisms of NEU antagonism.

Which mechanisms of NMDAR desensitization would be most affected by NEU? Ca^{2+}-dependent NMDAR inactivation in charge of the slow component of desensitization does not require agonist binding or channel opening (Krupp *et al.* 1996) whereas the onset rate of glycine-insensitive desensitization responsible for the fast component of desensitization depends upon the concentration of agonist (Sather *et al.* 1990). NEU block is agonist-dependent and NR2B-specific. Thus, Ca^{2+}-dependent inactivation that is independent of agonist and selective for NR2A receptors (Krupp *et al.* 1996) seems unlikely to be accountable for the enhancement of desensitization by NEU which is supported by the fact that a reduction of τ_{desen} by NEU did not depend on $Ca^{2+}{}_{ex}$ (Fig. 4b). Glycine-insensitive desensitization itself is not sensitive to the concentration of $Ca^{2+}{}_{ex}$ (Tong and Jahr 1994), but it is dramatically increased by $Ca^{2+}{}_{ex}$ in whole-cell recording mode (Legendre *et al.* 1993) as we observed. Accordingly, glycine-insensitive but Ca^{2+}-dependent desensitization, by which the peak of NMDA response is significantly controlled, is considered to be a major mechanism underlying NEU-enhanced desensitization. Although it is still premature to conclude whether NEU is selective only for NR2B receptors, it is quite certain that NEU has high affinity for NR2B compared with NR2A. This is further supported by the observation that NVP, an NR2A-specific blocker, does not suppress the initial peak responses of NMDAR, which are the targets for NEU action (Fig. 5a).

Collectively, we propose that the molecular mechanism for the inhibitory action of NEU on NMDAR is as follows. A high proportion of NMDARs occupies the desensitized states even in the absence of agonist (Sather *et al.* 1990), indicating that a substantial number of receptors are in a desensitized state. Binding of NEU to an NR2B-like site on the NMDAR enhances the stability of the closed states of the receptor channel, including receptors already desensitized even in the absence of NMDA, so that opening is less likely. Moreover, a progressive increase of the rate of transition from the active state to an agonist-associated desensitized state by NEU results in the acceleration and deepening of desensitization, which requires an intracellular Ca^{2+} process. The preferential binding of NEU to the agonist-associated desensitized state would likely contribute to the agonist-dependent nature of NEU inhibition.

Pharmacological significance of NEU as an NMDAR gating modifier

It is often impossible for competitive and non-competitive antagonists to distinguish between normal and excessive receptor activation, whereas uncompetitive molecules effectively antagonize excessive activation by toxic levels of glutamate. In this light, it is important to determine whether a drug such as NEU has an uncompetitive nature. Once bound to the activated receptor after channel opening, open-channel blockers with uncompetitive antagonism can usually be trapped within the closed channel, leading to an accumulation of block that makes them clinically unacceptable (MK-801 and phencyclidine). Gating modifiers with uncompetitive nature, such as ifenprodil and NEU, would be beneficial for clinical application in that their agonist-dependent nature comes from preferential binding to an agonist-associated closed state of the receptor, not from being trapped within channels. It is noteworthy that NEU exerts its own inhibitory action mainly through the enhancement of glycine-insensitive/Ca^{2+}-dependent desensitization. As Ca^{2+}-dependent activation of NMDAR desensitization serves to down-regulate the NMDAR component of synaptic currents at least transiently after repetitive synaptic stimulation and could limit further Ca^{2+} entry (Furukawa *et al.* 1995), this effect of NEU on NMDAR desensitization may be another advantage in alleviating side effects generated by NMDAR antagonists. In this regard, it is interesting to see that ifenprodil, unlike NEU, did not reduce the peak current when co-applied with NMDA (Fig. 5a).

Neu2000 exhibits a use-dependent block (Fig. 2b), however, its agonist use-dependency is not as prominent as an open-channel blockers, probably because of its fast off-rate. Notably, the unblocking rate of NEU (~600 ms) appears to

be almost eight times faster than that of memantine (~5000 ms; Chen *et al.* 1992), a well-known fast open-channel blocker. Low-affinity channel blockers with fast kinetics, such as amantadine, ketamine, and memantine, have a greater safety for therapeutic application than high affinity blockers (Kemp and McKernan 2002), probably because of their fast block and unblock time of NMDARs (Lipton 2004; Blanpied *et al.* 2005). Therefore, the rapid binding kinetics and low affinity of NEU are noteworthy. In addition, the lack of voltage-dependency of NEU may permit the inhibition of NMDA responses more effectively than open-channel blockers that usually decrease their blockade as the cells become depolarized, enhancing its protective potential against neurological insults where the prolonged depolarization occurs by excessive glutamate.

Very recently, it has been proposed that NR2B-specific antagonism may not be an optimal anti-excitotoxic strategy because NR2B-NMDARs can mediate both excitotoxic effects and pro-survival synaptic NMDARs signaling (Martel *et al.* 2009). However, it is still worth considering that a subtype-specific NMDAR antagonist acting extrasynaptically would have therapeutic potential for the following reasons. First, synaptic NMDARs have anti-apoptotic activity whereas extra-synaptic NMDARs contribute to excitotoxic cell death (Hardingham *et al.* 2002). Second, NR2B receptors are certainly present extrasynaptically, and NR2A receptors are incorporated into synapses during development (Liu *et al.* 2004; Thomas *et al.* 2006), despite the controversial evidence about localization of NR2A and NR2B receptors at synaptic and/or extra-synaptic sites (Li *et al.* 2002; Liu *et al.* 2007; Miwa *et al.* 2008). Third, short burst activity activates extra-synaptic NMDARs, and high frequency stimulation recruits additional extra-synaptic receptors (Harris and Pettit 2007). Therefore, it is highly possible that excessive glutamate spilled over in a synapse during ischemic insult recruits extrasynaptic NMDARs, which are mainly composed of NR2B subunits. An NR2B inhibitor would be better than either an NR2A inhibitor or enhancer (Liu *et al.* 2007) in protecting cells from excitotoxicity, although both NR2A and NR2B can elicit excitotoxicity. We thus expect that NEU which has a relatively high affinity for NR2B receptor may inhibit NMDA response with only minor interference in normal synaptic transmission.

Neu2000 is an NMDAR gating modifier with an uncompetitive antagonism mechanism. The acceleration of Ca^{2+}-dependent desensitization, a negative feedback mechanism to prevent the undesirable effects of excessive activation, by NEU should be an important factor for preventing neurons from glutamate neurotoxicity. Moreover, its specificity for NR2B as well as low affinity/fast kinetics seems likely to mitigate its undesirable side-effects. Notably, NEU performs these actions at much lower concentrations than sulfasalazine within the therapeutic window (Ryu *et al.* 2003). Therefore, we propose that NEU, which may be extremely effective with better side-effect profiles in mitigating *in vivo* ischemic damage, is an attractive pharmacological candidate for future drug development. As NR2B-specific antagonists such as ifenprodil and CP-101606 exhibit efficacy in pre-clinical pain models (Chizh and Headley 2005), NEU may be effective even for chronic pain treatments. Clinical tests will prove these in the near future.

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