Indistinguishable Synaptic Pharmacodynamics of the N-Methyl-D-Aspartate Receptor Channel Blockers Memantine and Ketamine

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ABSTRACT

Memantine and ketamine, voltage- and activation-dependent channel blockers of N-methyl-D-aspartate (NMDA) receptors (NMDARs), have enjoyed a recent resurgence in clinical interest. Steady-state pharmacodynamic differences between these blockers have been reported, but it is unclear whether the compounds differentially affect dynamic physiologic signaling. In this study, we explored nonequilibrium conditions relevant to synaptic transmission in hippocampal networks in dissociated culture and hippocampal slices. Equimolar memantine and ketamine had indistinguishable effects on the following measures: steady-state NMDA currents, NMDAR excitatory postsynaptic current (EPSC) decay kinetics, progressive EPSC inhibition during repetitive stimulation, and extrasynaptic NMDAR inhibition. Therapeutic drug efficacy and tolerability of memantine have been attributed to fast (EPSC) decay kinetics, progressive EPSC inhibition during repetitive state NMDA currents, NMDAR excitatory postsynaptic current (NMDA) receptor channel blockers. Memantine is neuroprotective voltage-dependent blocker re-equilibration with several experimental manipulations of gating efficacy. Excitatory postsynaptic potential-like voltage commands produced drug differences only with large, prolonged depolarizations unlikely to be attained physiologically. In fact, we found no difference between drugs on measures of spontaneous network activity or acute effects on plasticity in hippocampal slices. Despite indistinguishable synaptic pharmacodynamics, ketamine provided significantly greater neuroprotection from damage induced by oxygen glucose deprivation, consistent with the idea that under extreme depolarizing conditions, the biophysical difference between drugs becomes detectable. We conclude that despite subtle differences in voltage dependence, during physiologic activity, blocker pharmacodynamics are largely indistinguishable and largely voltage independent.

The drugs are pharmacologically similar, but the literature suggests small steady-state differences. Because physiologic activity is not inherently steady-state, we explored cellular and receptor-level drug differences under dynamic conditions. Ketamine and memantine both require channel opening for access to and for exit from the NMDAR channel (Parsons et al., 1993; Lipton, 2005; Johnson and Kotermanski, 2006; Gilling et al., 2009; Kotermanski et al., 2009). During sustained, steady-state agonist presentation, memantine may display slightly faster kinetics of block than ketamine (Gilling et al., 2009). Further, memantine has been found to bind two sites with differing dependence on channel opening, leading to partial trapping upon channel closure. Meanwhile, ketamine exhibits full trapping (Blanpied et al., 1997; Gilling et al., 2009; Kotermanski et al., 2009), although there is some evidence for two ketamine sites (Orser et al., 1997). Contrary to

Introduction

Memantine and ketamine are activation-dependent and voltage-dependent N-methyl-D-aspartate (NMDA) receptor (NMDAR) channel blockers. Memantine is neuroprotective and is used to enhance cognition in dementia (Ditzerl, 1991; Lipton, 2005). Ketamine is an anesthetic and analgesic with psychotomimetic effects and abuse potential (Krystal et al., 1994), but it has drawn recent attention as a fast-acting antidepressant (Zarate et al., 2006; Aan Het Rot et al., 2012).

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ABBREVIATIONS: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid; d-APV, d-2-amino-5-phosphonovalerate; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; MEA, multielectrode array; MK-801, 6-(+)s-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NBOX, 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; OGD, oxygen glucose deprivation; P_sp, open probability.
agonist presentation in most previous studies, physiologic, synaptic agonist presentation is brief and transient (Lester et al., 1990; Clements et al., 1992). This non–steady-state profile of agonist presentation could influence activation-dependent channel blockers and accentuate any pharmacodynamic differences. Memantine might be a selective antagonist for extrasynaptic NMDAR populations (Okamoto et al., 2009; Xia et al., 2010; Wroge et al., 2012). However, whether this effect is secondary to the more sustained agonist presentation at extrasynaptic versus synaptic receptors is unclear, and whether ketamine shares any extrasynaptic receptor selectivity has not been explored.

Both drugs are also voltage dependent. At sustained positive potentials, memantine appears to exhibit weaker block (more voltage dependence) than ketamine (Gilling et al., 2009). Depolarization, like agonist presentation, is rarely sustained during physiologic activity except under pathophysiologic conditions. How transient depolarization interacts with NMDAR blocker actions is unclear. Sufficiently rapid dissociation during excitatory postsynaptic potentials (EPSPs) could preserve transient synaptic transmission while blocking low-level, tonic extrasynaptic activation (Lipton, 2005; Gilling et al., 2009). On the other hand, if drug re-equilibration is slow relative to EPSP duration, drugs may have largely voltage-independent effects except under the most extreme conditions.

Our study focused on nonequilibrium agonist presentation and rapid voltage changes applicable to synaptic transmission. In a number of paradigms, the drugs exhibit remarkable similarities. The sole distinction observed was a difference in the apparent voltage dependence of the drugs in whole-cell, voltage-pulse experiments. Simulations revealed that the apparent voltage dependence could result from differences in channel opening and closing rates while blocker is bound and need not involve changes in microscopic voltage dependence of drug action. The difference between drugs did not manifest under most physiologic conditions because of the low open probability of the NMDAR channel (Rosenmund et al., 1995; Chen et al., 1999), which did not allow sufficient opportunity for dissociation of either drug during transient, synaptic-like depolarizations. The drugs also behaved similarly in their effects on spontaneous network activity and induction of plasticity. Differences emerged only under extreme depolarization during oxygen and glucose deprivation, during which memantine proved the weaker neuroprotectant.

Materials and Methods

Cell Cultures. Hippocampal cultures were prepared as either mass cultures or microcultures (as indicated in figure legends) from postnatal day 1 to 3; male and female rat pups were anesthetized with isoflurane, under protocols consistent with National Institutes of Health guidelines and approved by the Washington University Animal Studies Committee. Methods were adapted from earlier descriptions (Huetten and Baughman, 1986; Bekkers et al., 1990; Tong and Jahr, 1994; Mennerick et al., 1995). Hippocampal slices (500–1000 μm thickness) were digested with 1 mg ml−1 papain in oxygenated Leibovitz L-15 medium (Life Technologies, Gaithersburg, MD). Tissue was mechanically triturated in modified Eagle’s medium (Life Technologies) containing 5% horse serum, 5% fetal calf serum, 17 mM d-glucose, 400 μM glutamine, 50 U ml−1 penicillin, and 50 μg ml−1 streptomycin. Cells were seeded in modified Eagle’s medium at a density of ~650 cells mm−2 as mass cultures (onto 25-mm cover glasses coated with 5 mg ml−1 collagen or 0.1 mg ml−1 poly-L-lysine with 1 mg ml−1 laminin) or 100 cells mm−2 as “microisland” cultures (onto 35-mm plastic culture dishes coated with collagen microdroplets on a layer of 0.15% agarose). Cultures were incubated at 37°C in a humidified chamber with 5% CO2/95% air. Cytosine arabinoside (6.7 μM) was added 3 to 4 days after plating to inhibit glial proliferation. The following day, half of the culture medium was replaced with Neurobasal medium (Life Technologies) plus B27 supplement (Life Technologies).

Human Embryonic Kidney Cell Transfection. Human embryonic kidney cell line (HEK293) cells were grown and split when 50–75% confluent in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1 mM glutamine with penicillin or streptomycin. One day before transfection, cells were split and plated directly on 35-mm plastic dishes. When 30–50% confluent, cells were transferred to Opti-Med transfection media and transfected using Lipofectamine 2000 with glutamate receptor subunits GluN1 and GluN2A or GluN2B in a 1:3 ratio. GluN subunit DNA constructs were gifts of Drs. Elias Aizenman and Jon Johnson (University of Pittsburgh). Green fluorescent protein–positive HEK cells were recorded 2–5 days post transfection. For recordings, cells were transferred to an extracellular (bath) solution containing (in mM): 138 NaCl, 4 KCl, 2 CaCl2, 10 glucose, 10 HEPES, and 0.5 μM D-2-amino-5-phosphonovalerate (D-APV), pH 7.25, adjusted with NaOH. Solutions were perfused over the cells using a gravity-driven local perfusion system from a common tip. The estimated solution exchange times were <100 milliseconds (10–90% increase), estimated from junction current rises at the tip of an open patch pipette. For synaptic recordings, these solutions contained (in mM) 0.01 glycine, 0.001 NBQX (2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline), and 0.025 bicuculline methobromide and contained no D-APV. For exogenous NMDA application during voltage perturbations, 0.25 mM CaCl2 was used (in APV-free perfusion solutions) to minimize Ca2+-dependent NMDAR desensitization (Zorumski et al., 1989), and 250 μM tetrodotoxin was added to prevent network activity. During network activity studies (Fig. 9), all blockers were eliminated and 2 mM Ca2+, 1 mM Mg2+, and 1 μM glycine were used. During HEK cell recordings, antagonists and tetrodotoxin were omitted. Unless otherwise noted, exogenous NMDA concentration was 300 μM. In experiments using tricine, 10 mM tricine was added to external solutions, and pH was adjusted to 7.25. The tip resistance of patch pipettes was 3–6 MΩ when filled with an internal solution containing (in mM): 130 potassium gluconate, 0.5 CaCl2, 5 EGTA, 4 NaCl, and 10 HEPES at pH 7.25, adjusted with KOH. Potassium was used as the main cation for autaptic stimulation to preserve action potential waveform, but in experiments examining current responses to exogenous agonists, cesium methanesulfonate or cesium gluconate was used in place of potassium gluconate to block potassium channels and improve spatial voltage clamp quality. Holding voltage was typically ~70 mV unless otherwise noted. Access resistance (8–10 MΩ) was compensated 80%–100% for EPSC measurements. For evoked EPSCs, cells were stimulated...
with 1.5 millisecond pulses to 0 mV from −70 mV to evoke autaptic transmitter release (Mennerick et al., 1995).

Artificial EPSP (αEPSP) waveforms were generated in Excel using the following equation:

\[ V_n = V_{\text{in}} \left[ \frac{t}{\tau} \right] \exp \left( 1 - \frac{t}{\tau} \right) \]

\( V_{\text{in}} \) was set to values varying from 0 to +70 mV, yielding a maximum depolarization to −20 mV from the holding potential at −90 mV, and \( \tau \) was either 30 milliseconds (for short αEPSPs) or 300 milliseconds (for long αEPSPs). The resulting data were converted to a command waveform in Clampfit 10.1. Voltage-gated sodium currents were suppressed with 250 nM tetrodotoxin for this experiment.

For network activity analysis, mass cultures were seeded on multielectrode arrays (MEAs; Multichannel Systems) (Mennerick et al., 2010). Recordings were performed 15 days in vitro. For figures and statistics, effects of drugs over a 30-minute recording period were compared with the average of activity (30 minutes) before drug administration and after drug washout. Array-wide spike detection rate was measured as the total number of spikes across the entire array in each second of recording. Bursts were defined as three or more spikes on a single contact with an interspike interval less than a critical duration of 150 milliseconds (Wagenaar et al., 2006). Burst duration was the interval between the first spike and the last spike in a burst.

Hippocampal Slices. Slices were harvested from 28- to 32-day-old male albino rats under isoflurane anesthesia (Tokuda et al., 2010). For electrophysiology, hippocampal slices were transferred to a submersed recording chamber with continuous bath perfusion of artificial cerebrospinal fluid (124 NaCl, 5 KCl, 2 MgSO₄, 2 CaCl₂, 1.25 NaH₂PO₄, 22 NaHCO₃, 10 glucose, bubbled with 95% O₂/5% CO₂) at 2 ml/min at 30°C. Extracellular recordings were obtained from the apical dendritic layer of the CA1 region elicited with 0.1-millisecond constant current pulses through a bipolar stimulating electrode placed in stratum radiatum. EPSPs were monitored using a half-maximal stimulus based on a baseline input-output curve. After establishing a stable baseline, long-term potentiation (LTP) was induced by applying a single 100 Hz × 1 second high-frequency stimulus using the same intensity stimulus as used for monitoring. Long-term depression (LTD) was induced with 1-Hz low-frequency stimulation for 15 minutes. An input-output curve was repeated 60 minutes after induction protocols for statistical comparisons of changes in EPSP slopes at half-maximal intensity. Signals were digitized and analyzed using pCLAMP software (Molecular Devices, Union City, CA).

Oxygen Glucose Deprivation. Culture medium in mass cultures (13–14 days in vitro) was exchanged for minimum essential medium (catalog no. 11090-081; Life Technologies) with no added glucose or glycine, supplemented with 10 μM glycine immediately before hypoxia exposure in a commercially available chamber (Billups-Rothenberg, Inc., Del Mar, CA), humidified, and saturated with 95% nitrogen and 5% CO₂ at 37°C for 2.5 hours. Drugs were added directly to culture medium before oxygen-glucose deprivation (OGD). The gas exchange followed the specifications of the chamber manufacturer (flow of 20 l/min for 4 minutes to achieve 100% gas exchange). After OGD, cells were returned to their original medium and incubated under standard culture conditions until the cell death assay (24 hours later). We used Hoechst 33342 (5 μM) to identify all nuclei and propidium iodide (3 μM) for 30 minutes to stain nuclei of cells with compromised membranes. Five 10 × microscope fields were quantified per condition per experiment, yielding >100 total neurons for each condition. Ratios of healthy neurons were quantified as the fraction of propidium iodide–negative neuronal nuclei to total neuronal nuclei. Automated cell-counting algorithms (Imaged Software; NIH, Bethesda, MD) were used for cell counts. Toxicity experiments were treated as a dependent sample design, in which sibling cultures plated in identical media lost and exposed to hypoxia at the same time were compared by repeated measures statistics.

Simulations. NEURON software (Carnevale and Hines, 2006) was used to generate simulations of voltage step perturbations. The kinetic scheme used for the NMDAR, and rate constants were adapted from previous work (Blanpied et al., 1997).

Data Analysis. Electrophysiology data acquisition and analysis were performed primarily using pCLAMP 10 software (Molecular Devices). MEA data were analyzed with Igor Pro (WaveMetrics, Lake Oswego, OR). All electrophysiologic measurements were processed with Microsoft Excel and are presented as mean ± S.E.M. Statistical significance was determined using a Student’s one-tailed or two-tailed t test, unless indicated otherwise, with a Bonferroni correction for multiple comparisons where appropriate, and significance was taken as \( P < 0.05 \). One-tailed tests were reserved for later experiments on network function and toxicity, after a hypothesis about the direction of difference between memantine and ketamine emerged based on the difference in voltage dependence between drugs. Data plotting, statistical analysis, and curve fitting to the Hill equation were performed with SigmaPlot (Systat, San Jose, CA). Figure preparation was performed in SigmaPlot and Adobe Photoshop (San Jose, CA).

Decay time constants were measured using standard exponential fitting functions using a least-squares minimization algorithm or a Chebyshev transform in pCLAMP software.

Materials. All drugs were obtained from Sigma-Aldrich (St. Louis, MO) except for D-APV and NBQX (Tocris Bioscience, Minneapolis, MN). Culture media were obtained from Life Technologies (Grand Island, NY).

Results

Indistinguishable Modulation of NMDAR EPSCs by Memantine and Ketamine at Constant Voltage. To aid the choice of ketamine and memantine concentrations for our studies, we examined drug effects during sustained NMDA application in cultured dissociated hippocampal neurons (Fig. 1, A and B). Consistent with other studies (Gilling et al., 2009; Kotermanski and Johnson, 2009; Traynelis et al., 2010), both drugs behaved similarly at equimolar concentrations. Although memantine’s IC₅₀ was 2.1 μM and ketamine’s was 1.5 μM, these values were statistically indistinguishable (Fig. 1C). The values are likely a slight overestimate because block did not always reach a steady state at low antagonist concentrations. However, in subsequent experiments, equimolar concentrations inevitably yielded indistinguishable steady-state effects at negative membrane potentials, even with prolonged blocker application.

As use-dependent NMDAR antagonists, both memantine and ketamine require NMDAR channel activation to block the channel. Thus, the temporal parameters of NMDAR activation strongly affect the blocking ability of memantine and ketamine. During steady-state receptor activation, the blocker equilibrates with its binding site, and kinetic differences between blockers may be masked. Steady-state agonist presentation also poorly recapitulates physiologic events where agonist presentation is brief and NMDAR activation is transient (Lester et al., 1990; Clements et al., 1992). To investigate whether memantine and ketamine exhibit kinetic differences that result in differential modulation of the NMDAR during synaptic activity, we measured evoked NMDA-EPSC amplitude and decay kinetics in the presence of drug. Drugs were applied using an interleaved protocol, and EPSCs were allowed to recover to baseline between drug applications. With 20-second drug preapplication that persisted through EPSC stimulation, we found that both drugs equivalently accelerated EPSC decay (Fig. 2A). As previously observed, NMDA EPSC kinetics exhibited biexponential decay (Lester...
et al., 1990). Both time constants of decay were accelerated in the presence of memantine or ketamine, and the contribution of the fast component of decay increased. No detectable difference was found between equimolar memantine and ketamine in any of these properties (Fig. 2B).

If memantine and ketamine differ in their ability to dissociate from closed channels, the drugs may have differing effects on peak EPSC amplitudes during intermittent, repetitive synaptic agonist presentation. To test this, we evoked NMDAR EPSCs continuously at a frequency of 0.04 Hz to allow complete recovery from presynaptic, frequency-dependent facilitation and depression (Mennerick et al., 1995). We found that the time course of progressive block by ketamine and memantine during this continuous stimulation was indistinguishable (Fig. 3). The similarity of progressive block by memantine and ketamine suggests similar binding rates for the two drugs at −70 mV.

Memantine has been described as a selective blocker of extrasynaptic NMDARs over synaptic NMDARs compared with the very slowly reversible channel blocker MK-801.
Nonequilibrium Effects of Ketamine and Memantine

We examined the time course of the approach toward a new (reduced) steady-state level of inhibition at +50 mV (time course of re-equilibration) by generating time-resolved inhibition plots (Frankiewicz et al., 1996) created by dividing the leak-subtracted current trace in the presence of blocker by the leak-subtracted trace in the presence of NMDA alone and displayed as a percentage. The time course of blocker re-equilibration was biexponential for both drugs (Fig. 5, A1 and A2, lower panels), and the fast and slow time constants were indistinguishable (Fig. 5, A and C). However, memantine’s fast component of re-equilibration was slightly but significantly more prominent than ketamine’s (Fig. 5C, right). Consistent with the faster kinetics of memantine, weighted time constants for the return pulse to –70 mV also differed (0.8 ± 0.07 seconds for memantine, 1.3 ± 0.06 seconds for ketamine; P < 0.05).

In summary, voltage-pulse relaxation analyses uncovered two interesting aspects of drug actions that we pursued further in subsequent experiments. First, the data above yielded blocker re-equilibration weighted time constants of 1.2 ± 0.2 seconds for memantine and 2.1 ± 0.6 seconds for ketamine at +50 mV. This slow overall time course of re-equilibration for both drugs was surprising and seems in contrast to the previous suggestion that fast kinetics may permit normal synaptic transmission and therapeutic tolerability (Lipton, 2005). We explored the underlying mechanism of slow voltage...
Memantine and glycine receptors possess peak current amplitude depending on experimental conditions and subunit composition. Low channel gating efficacy at full agonist occupancy with peak open probability ($P_{\text{open}}$) 0.04–0.35, depending on experimental conditions and subunit composition (Rosenmund et al., 1995; Chen et al., 1999). In contrast, nicotinic and glycine receptors possess peak $P_{\text{open}}$ near 0.9 (Jin et al., 2009). Because channel opening is required for blocker binding and dissociation (Chen and Lipton, 1997), we reasoned that the slow re-equilibration might result from this low $P_{\text{open}}$ rather than slow drug binding/dissociation kinetics. A low gating efficacy might limit the rate of memantine and ketamine re-equilibration after a step perturbation of voltage.

To test this possibility, we simulated a voltage-pulse protocol. We used a kinetic scheme from previous work (Blanpied et al., 1997) and previously established rate constants (Fig. 6A). The scheme accommodates previous observations of low agonist efficacy and gating of the blocked channel (Blanpied et al., 1997) but neglects desensitization and multiple open and closed states (Popescu and Auerbach, 2003). Although simplified, the model has fewer free parameters than more complex models, and it has proven useful in prior analyses. Thus, it represents a useful heuristic tool for our subsequent experimental tests. Simulation in the absence of blocker resulted in a $P_{\text{open}}$ of 0.03, similar to experimentally derived values for hippocampal neurons (Rosenmund et al., 1995). Because NMDAR channel gating exhibits only weak inherent voltage sensitivity (Jahr and Stevens, 1987; Clarke and Johnston, 2008), voltage dependence of gating was not incorporated into the model, and $P_{\text{open}}$ remained constant at both −70 and +50 mV, but the change in driving force was simulated (Fig. 6B, black trace).

We simulated the effect of 10 μM blocker with binding properties given in Fig. 6B legend (gray trace). The voltage dependence of the blocker was simulated by altering its $k_{\text{off}}$ value e-fold per 31.5 mV (Kotermanski and Johnson, 2009). Inhibition at −70 mV was relieved after a pulse to +50 mV with a single-exponential τ of 1.4 seconds. To test the effect of gating efficacy on re-equilibration, we altered efficacy by increasing $\beta_1$ and $\beta_2$ 10-fold. This manipulation caused the relaxation time constant during a step to +50 mV to decrease approximately 10-fold to 163 milliseconds (Fig. 6B, red trace, and C). A further increase in efficacy (1000-fold increase in opening rate) yielded a $P_{\text{open}} > 0.9$, and the resulting relaxation time constant was consistent with drug binding kinetics (Fig. 6C, τ = 0.002 second, equivalent to 1/($k_{\text{on}} + k_{\text{off}}$)). Although performed with a simplified gating scheme, these simulations demonstrate in principle that inefficient gating rate limits the re-equilibration of drug. Previous investigations have verified the assumptions of high $P_{\text{open}}$ for voltage-pulse relaxation analysis of other channels (Adams, 1977; Jin et al., 2009), but binding and dissociation rate constants for low $P_{\text{open}}$ NMDARs cannot be directly derived from whole-cell, voltage-pulse relaxations.

Another possible explanation for the slow time course of unblock could be slow dissociation kinetics of the drugs. We simulated the effect of altering $k_{\text{off}}$ for the blocker 10-fold, which resulted in substantially less steady-state block but little change in the time course of re-equilibration at positive voltages (Fig. 6B, blue trace). We conclude, therefore, that drug unbinding kinetics likely does not explain the slow time constants.
Nonequilibrium Effects of Ketamine and Memantine

Fig. 6. Simulations demonstrate that low $P_{\text{open}}$ slows voltage-dependent re-equilibration of blocker. (A) Kinetic scheme used in the simulations. Rate constants were adapted from Blanpied et al. (1997) and were $k_\alpha = 2 \mu \text{M}^{-1} \text{s}^{-1}, k_\beta = 40 \text{sec}^{-1}, \beta = 5.2 \text{sec}^{-1}, a = 130 \text{sec}^{-1}, k_{\text{off}} = 14.9 \mu \text{M}^{-1} \text{s}^{-1}, k_{\text{on}} = 7.6 \text{sec}^{-1}, \beta' = 0.3 \text{sec}^{-1}, a' = 35.4 \text{sec}^{-1}, k_{\text{off}'} = 0.2 \mu \text{M}^{-1} \text{s}^{-1}, k_{\text{on}'} = 0.02 \text{sec}^{-1}, [\text{NMDA}] = 300 \mu \text{M} \text{, } [\text{blocker}] = 10 \mu \text{M}. A2R^* \text{ and } A2R' \text{ represent the open channel in its unblocked and blocked state, respectively.} \text{ (B) Output of the simulation replicating a voltage jump in the presence (gray) and absence (black) of a voltage-dependent open-channel blocker of the NMDAR. Voltage dependence of the open channel blocker was achieved by decreasing } k_{\text{off}} \text{ by e-fold per 31.5 mV (Kotermanski and Johnson, 2009). Colored traces are as depicted in the legend. See Materials and Methods for more details of the simulation.} \text{ Simulation output (representing channels in the A2R^* state) was normalized to initial NMDA-only amplitude (3.8% of receptors for the black, gray, and blue traces; 28.6% for the red trace). Changing blocker dissociation (blue) reduced steady-state block but did not appreciably alter the re-equilibration kinetics at +50 mV. By contrast, accelerating channel opening (red) sped re-equilibration kinetics during wash off at -70 mV and after the pulse to +50 mV.} \text{ (C) Calculated rate constants derived from tau of re-equilibration at positive potentials at varying efficacies, plotted as a function of } P_{\text{open}} \text{ (altered by incrementing } \beta \text{ and } \beta' \text{ in 10-fold steps). Simulation output (black line) is compared with simulation input (gray line). } P_{\text{open}} \text{ needed to approach 1 (0.97) to retrieve values of the same order of magnitude of simulation input. Calculated rate constants for memantine from our data in Fig. 5 are also plotted as red lines. Horizontal lines are for reference purposes and are not a function of } P_{\text{open}}. \text{ Experimentally Increasing Gating Efficacy Speeds Blocker Re-Equilibration. Our modeling predicts that increasing } P_{\text{open}} \text{ should speed re-equilibration of channel blockers at positive potentials. To test this in our neuronal population, we manipulated levels of extracellular glycine. As a necessary coagonist, glycine levels control NMDAR gating. We found that decreasing bath glycine levels from 10 to 0.2 } \mu \text{M significantly slowed memantine re-equilibration (10 } \mu \text{M versus 0.2 } \mu \text{M glycine: weighted } 0.97 \pm 0.15 \text{ seconds versus } 2.8 \pm 0.3 \text{ seconds}; n = 8, P < 0.05). \text{ To test this further, we took advantage of the fact that diheteromeric GluN1/GluN2A NMDARs have higher efficacy than diheteromeric GluN1/GluN2B receptors (Chen et al., 1999). These subunits are present in nearly all NMDARs in cultured hippocampal neurons (Tovar and Westbrook, 1999). We heterologously expressed GluN2A or GluN2B, along with GluN1, subunits in HEK293 cells and measured the time course of memantine re-equilibration at +50 mV in the presence of 300 } \mu \text{M NMDA. As expected, if gating kinetics rate limits blocker behavior, we found that memantine re-equilibration at positive potentials was significantly faster in NMDARs containing GluN2A subunits (468 } \pm 67 \text{ milliseconds, weighted } \tau \text{) than in those containing GluN2B subunits (709.3 } \pm 94.4 \text{ milliseconds weighted } \tau; P < 0.05, \text{ Student's t test, } n = 20–22 \text{ (Fig. 7, A and B). Ketamine retained its property of slightly slowed voltage-dependent re-equilibration compared with memantine at both GluN2 isofoms (770 } \pm 146 \text{ milliseconds for GluN2A, 1727 } \pm 334 \text{ milliseconds for GluN2B; } P < 0.05 \text{ at each). Steady-state inhibition for memantine at -70 mV of GluN2A-containing versus GluN2B-containing receptors was 93% } \pm 1\% \text{ versus 98% } \pm 1\% \text{ (} P < 0.05, n = 20–22 \text{). The slight difference we found is consistent with previous work showing that memantine is weakly selective for GluN2B receptors with an IC50 ~2-fold greater for GluN2A over GluN2B populations (Kotermanski and Johnson, 2009). When memantine and ketamine were compared on the same GluN2A or GluN2B-bearing cell, both drugs displayed a weak preference for GluN2B-containing receptors (GluN2A versus GluN2B: memantine: 90% } \pm 2\% \text{ versus 97% } \pm 2\%; P < 0.05, \text{ and ketamine: 92% } \pm 2\% \text{ versus 98% } \pm 2\%; P < 0.05, n = 7), once again highlighting very similar effects of the two channel blockers. As a more direct and sensitive test of the hypothesis that gating efficacy dictates the slow re-equilibration, we took advantage of selective inhibition of GluN2A-containing receptors by low (nanomolar) concentrations of ambient zinc, which decreases open probability of GluN2A receptors, likely by affecting gating efficacy (Paoletti et al., 1997; Erreger and Traynelis, 2008; Gielen et al., 2009; Amico-Ruvio et al., 2011). We buffered ambient zinc in our extracellular bath solution using 10 mM tricine to relieve the effects of contaminating Zn2+ and found that NMDA responses from GluN2A, but not GluN2B, currents were significantly potentiated (71 } \pm 13\% \text{ increase in GluN2A, } n = 8, P < 0.05 \text{ versus } 9\% \pm 2\%; n = 8, P > 0.05 \text{ in GluN2B; Fig. 6, C1 and D1). Correspondingly, we found acceleration of memantine re-equilibration time course at +50 mV in the presence of tricine in GluN2A receptors (487 } \pm 334 \text{ milliseconds in the absence of tricine versus } 135 } \pm 26 \text{ milliseconds in the presence of tricine, } n = 8, P < 0.05; \text{ Fig. 7, C2 and D2). Blocker re-equilibration at GluN2B NMDARs was not significantly affected by tricine (545 } \pm 61 \text{ ms in the absence of tricine versus } 630 } \pm 135 \text{ milliseconds in the presence of tricine, } n = 8; \text{ Fig. 7, C2 and D2).}
Taken together, our data strongly suggest that the low \( P_{\text{open}} \) of the fully liganded NMDAR rate limits blocker re-equilibration, effectively trapping memantine and ketamine in the channel despite high agonist concentration.

**Memantine and Ketamine Show Little Unblocking during Mild-to-Moderate Artificial EPSPs.** We found that memantine exhibited a stronger fast component of relaxation (62% versus 48%) after a pulse to \( +150 \) mV compared with ketamine (Fig. 5). Because both blockers re-equilibrate slowly after strong depolarization, it is unclear what impact this subtle drug difference would have during EPSPs. Synaptic depolarizations are briefer and smaller than the voltage pulses to \( +150 \) mV and therefore may not elicit the drug difference observed in Fig. 5. To explore the size and duration of EPSPs necessary to elicit a difference between drug actions, we designed a voltage-command waveform that mimicked EPSPs of different amplitude and duration (see Materials and Methods). By examining current responses in the presence of \( 300 \mu M \) NMDA alone and in the presence of NMDA plus \( 2 \mu M \) memantine or ketamine, we created inhibition plots analogous to those in Figs. 5 and 7. We found that at mild to moderate depolarizations (up to a peak voltage of \( -55 \) mV), memantine and ketamine displayed little unblock and acted very similarly during short-duration \( \alpha \)EPSPs (Fig. 8C1). Memantine unblock diverged strongly from that of ketamine only with strong, prolonged \( \alpha \)EPSPs (compare Fig. 8, C1 and C2). These data suggest that weak synaptic activity is insufficient to relieve synaptic memantine or ketamine block. However, stronger activity reveals drug differences consistent with observations from voltage-pulse experiments. It is possible that the prolonged strong \( \alpha \)EPSPs in this experiment mimic the effects of high-frequency nervous system activity. If so, we might expect that spontaneous network activity or high-frequency

Fig. 7. Experimentally increasing \( P_{\text{open}} \) speeds re-equilibration. (A and B) GluN2A-containing NMDA receptors exhibit faster re-equilibration than GluN2B-containing receptors. (C1 and D1) \( 10 \) mM Tricine potentiates NMDA currents in GluN1/GluN2A-transfected HEK cells but not GluN1/GluN2B cells. (C2 and D2) Tricine speeds re-equilibration of memantine at positive voltages in GluN2A-containing but not GluN2B-containing receptors. See Results for quantification.

Fig. 8. Differences in voltage dependence are detectable only with large, broad depolarizations. (A1 and A2) Hippocampal neurons were voltage clamped using an \( \alpha \)EPSP voltage command waveform of short (A1, \( \tau = 30 \) milliseconds) or long (A2, \( \tau = 300 \) milliseconds) duration. Cells were bathed in saline, NMDA alone (\( 300 \mu M \)), then memantine (\( 2 \mu M \)) plus NMDA. In each condition, from a \( V_m \) of \(-90 \) mV, consecutive sweeps simulated EPSPs of increasing amplitude (\( V_m \) at maximum: \(-72.5, -55, -37.5, -20 \) mV). Leak currents in saline were subtracted from each experimental condition. Traces represent percent inhibition of the NMDA-only current during the smallest depolarization (\( -72.5 \) mV, dark red) and largest (\( -20 \) mV, bright red), so upward excursion of the traces represents relief from drug-induced inhibition. (B1 and B2) Same protocol for ketamine (\( 2 \mu M \)) (traces, dark green: \(-72.5 \) mV, bright green: \(-20 \) mV). (C1 and C2) Peak block at each potential for each EPSP duration for memantine and ketamine. Colored circles correspond to the conditions represented by the traces in A and B. No significant interaction was found between drug and voltage with the brief \( \alpha \)EPSPs [C1, two-way analysis of variance (ANOVA)]. However a significant interaction emerged with prolonged \( \alpha \)EPSPs, with memantine exhibiting more unblock than ketamine on strong depolarization (C2, \( *P < 0.05 \), drug by voltage interaction, two-way ANOVA, \( n = 5 \) to 6). n.s., not significant.
stimulation used to induce LTP might be differentially affected by memantine and ketamine. These ideas were tested in subsequent experiments.

Memantine and Ketamine Suppress Network Activity Similarly but Diverge in Neuroprotective Ability with Extreme Depolarization. Our aEPSP data suggest that memantine may more effectively escape the NMDAR channel during strong depolarization than does ketamine, but it is unclear whether physiologic activity achieves sufficiently prolonged, strong depolarization to reveal a drug difference, even with temporal summation of EPSPs and associated spiking. Furthermore, continuous agonist application inherent in the design of Fig. 8 may have facilitated drug dissociation during depolarization. To test directly whether the subtle difference in voltage dependence, apparent under the controlled conditions of Fig. 8, is detectable under conditions of physiologically transient depolarization and agonist presentation, we took a two-pronged approach. We examined the network effects of the channel blockers using both single-cell recording and network activity analysis using multielectrode arrays. First, using whole-cell recording in hippocampal cultures, we measured AMPAR-mediated, network-driven EPSCs as a measure of network activity (Fig. 9, A and B). Although NMDAR function was inhibited in the recorded cell by bath Mg²⁺ and voltage clamp to −70 mV, NMDARs in the surrounding network were free to contribute to spontaneous activity. We found that a low concentration (2 μM) of memantine and ketamine had negligible effects on network activity and did not differ from each other (Fig. 9B). In part, the weak effects could be due to the presence of physiologic Mg²⁺ and glycine concentrations (1 mM and 1 μM, respectively). Given that the weak effects of the antagonists may render differences indiscernible, we increased the antagonist concentration to 10 μM. At this concentration, both drugs suppressed network activity by ~30% (memantine: 25% ± 6%, ketamine: 32% ± 7%, n = 12; Fig. 9B), but again we could detect no differences between the drugs (P > 0.05 paired t test; Fig. 9B).

As a second approach, we measured network activity using MEAs to monitor spiking across the network. MEA recordings permitted us to examine network synchrony and spatial relationships not captured by single-cell recordings. These recordings were performed at 37°C and with divalent cations present to mimic physiologic conditions. We recorded network activity in the presence of 10 μM memantine or ketamine relative to baseline activity recorded before drug application and after drug washout (Fig. 9C). Both drugs strongly suppressed network activity as measured by array-wide spike detection rate and measures of bursting (Fig. 9D). Effects of the two drugs covaried indistinguishably across all measured parameters.

To explore effects in more intact networks, we examined the effects of ketamine and memantine on synaptic plasticity in hippocampal slices. At concentrations up to 10 μM, neither drug acutely affected the induction of LTP elicited by 100 Hz stimulation for 1 second (Fig. 10A). On the other hand, both drugs at concentrations of 1 and 10 μM inhibited LTD induction, evoked by prolonged 1-Hz low-frequency stimulation (Fig. 10B). The difference in effects between the two forms of NMDAR-induced plasticity could result from differences in stimulation parameters or other factors (see Discussion). Regardless, these results extend the profile of similar pharmacodynamics between the two drugs to well-established protocols of plasticity induction and highlight another nonsteady-state condition (repetitive stimulation) in which the drugs behave quite similarly.

Although the biophysical differences between memantine and ketamine were not evident during three assays of physiologic and stimulated activity, extreme conditions that evoke prolonged NMDAR activation might reveal differences between drugs as predicted by Fig. 8. To test this, we challenged cultured hippocampal neurons with OGD and evaluated the neuroprotective effects of memantine and ketamine. In this system, synaptic NMDARs play a dominant role in mediating excitotoxicity (Wroge et al., 2012). At the same concentration (10 μM) used for network studies, ketamine was significantly more neuroprotective than memantine was (Fig. 11). We conclude that under extreme conditions of pathophysiologic NMDAR activation, a pharmacodynamic difference between memantine and ketamine appears.

Discussion

Memantine and ketamine exhibit remarkably different clinical properties despite similar activity as NMDAR activation-dependent channel blockers. Our results suggest only slight pharmacodynamic differences in voltage dependence between drugs, even under nonsteady-state conditions, that do not become relevant until cells are challenged with pathophysiologic depolarization and NMDAR activation (OGD). Thus, pharmacokinetic and dosing differences may be more likely than pharmacodynamic differences to explain clinical differences (but see Kotermanski et al., 2013).

We aimed to determine whether the two drugs differ in nonequilibrium synaptic actions, which have not been explored in detail previously. Similarities in steady-state block may belie kinetic differences that could become more evident during non–steady-state conditions of physiologic activity. By analogy, the well-known calcium chelators EGTA and BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid] have similar Kd values but differ 100-fold in their koff and kon values. Differences between these buffers are not easily appreciated under equilibrium conditions. However, the faster buffer BAPTA much more effectively depresses synaptic vesicle release during nonequilibrium, depolarization-elicted Ca²⁺ influx (Rozov et al., 2001). In the case of memantine and ketamine, by contrast, steady-state similarities do not mask non–steady-state dissimilarities.

One pharmacodynamic difference may be a selective inhibition by memantine of extrasynaptic receptors (Okamoto et al., 2009; Xia et al., 2010). However, we found that memantine and ketamine have indistinguishable actions even at extrasynaptic NMDARs. Previous experiments arguing for a selective effect of memantine on extrasynaptic receptors compared memantine with the slowly dissociating open-channel blocker MK-801 (Xia et al., 2010), but our results demonstrate that memantine holds no extrasynaptic preference over ketamine. Any preference for extrasynaptic receptors is likely to result from the slow, sustained agonist presentation of ambient neurotransmitter at extrasynaptic receptors, rather than drug preference for certain receptor populations (Wroge et al., 2012).
Fig. 9. Network effects of memantine and ketamine. (A) A neuron was voltage clamped at ~70 mV in a blocker-free external solution containing 1 μM glycine. Network activity was measured as AMPA-driven spontaneous EPSCs onto the neuron over 60-second intervals. Network activity was allowed to stabilize for 2 minutes before baseline data collection, and drugs were allowed 60 seconds of equilibration before recording. Memantine and ketamine (10 μM) were interleaved between saline recordings, and the presentation order was reversed from cell to cell. (B) Synaptic activity was quantified as synaptic charge exceeding a threshold of 7.5 pA over the 60 seconds of recording time. Activity in drug was compared with the average activity during baseline and washout conditions. At 2 μM, no significant effect of either drug on synaptic activity (n = 6) was seen. However, at 10 μM, both drugs depressed activity (*P < 0.05, unpaired t test to normalized baseline), but depression did not significantly differ between drugs (n = 12). (C) MEA recordings from cultures in the presence of control media (baseline) or 10 μM memantine or ketamine. Representative raster plots are shown from one
A second hypothesis to explain memantine's therapeutic profile involves its rapid kinetics coupled with its strong voltage dependence. These properties are proposed to allow memantine to dissociate rapidly during transient synaptic depolarization and preserve synaptic transmission (Parsons et al., 1993; Frankiewicz et al., 1996; Lipton, 2005). During sudden voltage changes, contrary to our expectations and previous results (Frankiewicz et al., 1996), we found that memantine and ketamine both equilibrated slowly, suggesting little relief from block during synaptic depolarization and agonist presentation.

Our conclusion that the efficiency of channel gating affects the time course of relief from block during depolarization has implications for prior work investigating blocker kinetics. Past estimates of memantine binding kinetics differ by several orders of magnitude across different studies (Blanpied et al., 1997; Gilling et al., 2009). Our results provide a partial resolution. Studies using voltage pulses (Frankiewicz et al., 1996) or offset currents after drug washout (Gilling et al., 2009) will be susceptible to errors in $k_{on}/k_{off}$ estimates resulting from low gating efficacy. Some studies of other cell types have found that memantine re-equilibrates at positive potentials much faster than we observed (Frankiewicz et al., 1996). Although we do not have a complete explanation for this discrepancy, one possibility is that the receptors from different neuronal populations used in previous work exhibited higher efficacy, yielding faster apparent drug re-equilibration (Fig. 6).

Despite slow overall kinetics, memantine yielded faster voltage-dependent relief from block than ketamine and more steady-state relief from block with strong depolarization. What is the basis for this difference between drugs? We suggest that it may reflect differences in channel gating while drug is bound. Although the model that we used does not capture many complexities of NMDAR channel gating (Popescu and Auerbach, 2003), it allowed us to test whether differences in gating can in principle explain experimentally observed drug differences. We used the baseline blocking parameters from Fig. 6 to represent ketamine-like block. From this baseline, we attempted to recapitulate features of memantine block by doubling $\beta'$ (opening rate of blocker-bound NMDAR).

We first doubled $\alpha$' to maintain identical steady-state block at −70. This led to a speeding of time course of re-equilibration but no change in steady-state block at +50 mV (not shown). However, if $\alpha$' was increased by somewhat less, the simulated steady-state block at −70 mV was still very similar to baseline (likely experimentally indistinguishable; Fig. 12), the time course of re-equilibration was accelerated, and the apparently stronger steady-state voltage dependence of memantine was recapitulated (Fig. 12). Thus, by altering channel opening and closing with blocker bound, the two most important differences between memantine and ketamine in our experiments were captured. Note that the apparent voltage-dependent drug differences were mimicked without adjusting the microscopic voltage dependence (e-fold per 31.5 mV for both drugs) or microscopic kinetics of drug binding. Although this explanation for drug differences remains hypothetical, it is attractive in its simplicity and its reliance on the previously proposed nature of blocker action (Blanpied et al., 2005).

This difference in voltage dependence of the drugs was the sole difference detected, and we investigated its potential physiologic significance with four experiments: artificial EPSPs, two measures of network activity, and plasticity induction. These results suggest that acute effects of the drugs are indistinguishable acting on neuronal circuits in dissociated culture and brain slices. This conclusion holds for a wide range of temperatures (22°C for spontaneous EPSCs, 30°C for slices, and 37°C for MEA recordings) and under conditions in which divalent cation concentrations were near physiologic 1 to 2 mM (all studies of physiologic activity and toxicity). Thus, although we might expect voltage-dependent differences between memantine and ketamine to accumulate during spontaneous or evoked high-frequency activity, differences were not evident in our assays of network spiking or plasticity induction.

The differential effects on LTD versus LTP of both drugs could have several explanations. Differential effects on LTD could reflect selective inhibition of extrasynaptic NMDARs (Xia et al., 2010), which have been preferentially associated with LTD (Papouin et al., 2012). However, our previous work and results herein suggest little selectivity for extrasynaptic versus synaptic receptors in hippocampal preparations when agonist presentation is held constant (Wroge et al., 2012; but see Wild et al., 2013). LTD also appears to preferentially involve GluN2B-containing receptors (Liu et al., 2004; Izuimi et al., 2005, 2006). The slight preference of both ketamine and memantine for GluN2B-containing receptors (Fig. 7) could therefore participate in the differential block of LTD. Another contributor could be differences in induction protocols themselves (900 pulses over 900 seconds versus 100 pulses over 1 second). LTD induction is associated with less postsynaptic charge transfer than LTP induction (Dudek and Bear, 1992; Bear, 1995). This would seem to favor block during LTD induction. However, NMDAR responses during LTD are associated with less overall membrane depolarization, a condition favoring block. Tonic receptor activation (Sah et al., 1989; Povyshueva and Johnson, 2012) and associated accumulated block may also be greater during the longer blocker incubation associated with LTD.

Finally, LTP inhibition may require a greater fractional block of receptors than does LTD inhibition. Previous work showed that 4–6 hours of memantine incubation is required to inhibit LTP, with an IC50 of ~12 μM (Frankiewicz et al., 1996). It is unclear why such a long incubation should be required, as much briefer incubation was sufficient in our studies to block LTD, and blocker effects on spontaneous activity in MEA recordings inevitably equilibrated by <15 minutes. It is possible that channel block achieved early in incubation in the Frankiewicz et al. (1996) study resulted in complicated downstream signaling cascades, similar to the effects seen with ketamine (Auyt et al., 2011) and other NMDAR antagonists.
Regardless of the multifactorial nature of the differential effects of blockers on plasticity, the indistinguishable actions of the drugs during plasticity induction emphasize their remarkable similarity under varied conditions.

Despite statistical indistinguishability in most assays, ketamine proved more neuroprotective than did memantine against OGD damage. We previously showed that during in vitro OGD under our conditions, damage is mainly NMDAR-mediated (Hogins et al., 2011; Wroge et al., 2012). In the present experiments, we predicted that the sustained depolarization and receptor activation produced by OGD would reveal the difference in voltage dependence predicted from experiments described in Figs. 5 and 8. Indeed, memantine proved slightly less neuroprotective, presumably because of the slightly faster and more complete block relief with depolarization. The result is ironic since memantine is the clinically indicated neuroprotectant. If memantine is the better clinical neuroprotectant, off-target effects or other in vivo factors may explain the discrepancy with our results.

In summary, we find that the clinically important drugs memantine and ketamine are largely pharmacologically indistinguishable under both steady-state and nonequilibrium conditions. A slight difference in voltage dependence is the sole characteristic that distinguishes the drugs. Only under severe depolarizing conditions of OGD was this biophysical difference revealed as a neuroprotective difference between drugs.

(Zorumski and Izumi, 2012).


