Brief Communications

Memantine Preferentially Blocks Extrasynaptic over Synaptic NMDA Receptor Currents in Hippocampal Autapses

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Glutamate is the major excitatory neurotransmitter in the brain. The NMDA subtype of glutamate receptors (NMDAR) is known to mediate many physiological neural functions. However, excessive activation of NMDARs contributes to neuronal damage in various acute and chronic neurological disorders. To avoid unwanted adverse side effects, blockade of excessive NMDAR activity must therefore be achieved without affecting its physiological function. Memantine, an adamantane derivative, has been used for the treatment of Alzheimer's disease with an excellent clinical safety profile. We previously showed that memantine preferentially blocked neurotoxicity mediated by excessive NMDAR activity while relatively sparing normal neurotransmission, in part because of its uncompetitive antagonism with a fast off-rate. Here, using rat autaptic hippocampal microcultures, we show that memantine at therapeutic concentrations (1–10 μM) preferentially blocks extrasynaptic rather than synaptic currents mediated by NMDARs in the same neuron. We found that memantine blocks extrasynaptic NMDAR-mediated currents induced by bath application of 100 μM NMDA/10 μM glycine with a twofold higher potency than its blockade of the NMDAR component of evoked EPSCs ($EPSCs_{NMDAR}$); this effect persists under conditions of pathological depolarization in the presence of 1 mM extracellular Mg^{2+}. Thus, our findings provide the first unequivocal evidence to explain the tolerability of memantine based on differential extrasynaptic/synaptic receptor blockade. At therapeutic concentrations, memantine effectively blocks excessive extrasynaptic NMDAR-mediated currents, while relatively sparing normal synaptic activity.

Introduction

Excessive activation of ionotropic glutamate receptors by excitatory amino acids, such as glutamate, is thought to contribute to many neurological diseases, ranging from acute hypoxic-ischemic brain injury to chronic neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases, HIV-associated neurocognitive disorders, and amyotrophic lateral sclerosis (Choi, 1988; Lipton and Rosenberg, 1994; Lipton, 2006). In many CNS areas, the predominant form of this neurotoxicity appears to be mediated by overactivation of the NMDA subtype of glutamate receptor (NMDAR) and subsequent influx of excessive Ca^{2+} (Rothman and Olney, 1986; Choi et al., 1988; Chen et al., 1992). Since physiological NMDAR activity is very important for normal brain function in the nervous system, whereas receptor overstimulation contributes to disease states, blockade of excessive NMDAR activity must be achieved without interference of physiological function. High-affinity NMDAR antagonists are effective at NMDAR blockade, but toxic to neurons and produce neurobehavioral side effects at least in part because they block normal synaptic activity (Lipton, 1993; Rogawski, 1993; Rogawski and Wenk, 2003). In contrast, studies in our laboratory and subsequently others have shown that the adamantane derivative, memantine, preferentially blocks excessive NMDAR activity without disrupting physiological synaptic activity (Chen et al., 1992, 1998; Léveillé et al., 2008; Papadia et al., 2008; Okamoto et al., 2009). Memantine, at low micromolar concentrations, accomplishes this through its action as a low-affinity, uncompetitive, open-channel blocker with a relatively rapid off-rate from the channel (Chen and Lipton, 1997; Lipton, 2006, 2007). Phase 3 clinical trials have shown that memantine is effective in treating moderate-to-severe Alzheimer's disease while being well tolerated at doses that yield concentrations at the receptor approaching 10 μM (Reisberg et al., 2003; Tariot et al., 2004; Chen and Lipton, 2006; Okamoto et al., 2009).

The molecular mechanism underlying the clinical tolerability of memantine, however, remains somewhat contentious (Lipton, 1993; Rogawski and Wenk, 2003; Lipton and Chen, 2004; Lipton, 2006, 2007). According to its kinetics of interaction with NMDAR channels, we have predicted that memantine predominantly acts as an open-channel blocker in the presence of prolonged elevation of glutamate concentration (on the scale of minutes or longer), but is relatively inactive when glutamate is elevated for only milliseconds, as in synaptic transmission (Chen and Lipton, 1997; Lipton, 2006, 2007). We and others have previously shown that, unlike other NMDAR antagonists, meman-

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tine appears to be effective in blocking excessive extrasynaptic NMDAR activation by bath-applied NMDA, but less effective on synapses (Chen et al., 1992, 1998; Okamoto et al., 2009). Here, we directly quantify this phenomenon and for the first time show distinctive effects of low micromolar concentrations of memantine on synaptic and extrasynaptic NMDARs in the same preparation, the hippocampal autaptic neuron. We demonstrate that low concentrations of memantine act as an effective antagonist of excessive extrasynaptic NMDAR stimulation while manifesting much less effect on NMDAR-mediated synaptic activity.

Materials and Methods

Cell culture. Sprague Dawley rat pups of both genders were killed on postnatal day 0 to harvest hippocampal cells for generation of microisland cultures (Segal and Furshpan, 1990; Segal, 1991). This method was used to grow single, isolated excitatory hippocampal neurons that form autapses in culture. Briefly, a 0.15% agarose solution was spread thinly on 12 mm coverslips in 35 mm dishes and allowed to dry. Then a glass microatomizer (Kontes Glass) was used to spray onto the agarose a fine mist of a 17 mM acetic acid solution containing collagen at 0.0625 mg/ml and poly-D-lysine at 0.05 mg/ml (Sigma-Aldrich) to produce "collagen dots" on the agarose base. Hippocampal neurons were dissociated and plated onto the coated coverslips at $4-8 \times 10^4$ cells per dish.

Electrophysiology. EPSCs were recorded under voltage clamp from solitary neurons using a patch electrode after 13 d *in vitro*. Whole-cell recordings were performed with a cesium chloride-rich intracellular solution. Series resistance was compensated 70–80% using multiclamp 700A patch-clamp amplifier circuitry (Molecular Devices). Currents were acquired using a Digidata 1322 interface and pClamp 10.1 software. All recordings were made at room temperature at a holding potential (V_h) of −25 or −70 mV. EPSCs were evoked from $V_h = -70$ mV at a stimulation rate of 0.1 Hz by stepping to +80 mV for 1 ms; from $V_h = -25$ mV, the neurons were first subjected to a hyperpolarizing prepulse to −70 mV for 3 s to remove Na^+ channel inactivation, and then returned to −25 mV to elicit an EPSC. Currents were digitally sampled at 10–20 kHz and filtered at 2 kHz. The extracellular solution contained the following (in mM): 137 NaCl, 1 $NaHCO_3$, 0.34 Na_2HPO_4, 5.36 KCl, 0.44 KH_2PO_4, 3 $CaCl_2$, 5 HEPES, 22.2 glucose, 0.01 glycine, 0.003 strychnine, pH adjusted to 7.2. The intracellular solution contained the following (in mM): 120 CsCl, 20 tetraethylammonium chloride, 10 HEPES, 2.25 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 4 MgATP, 0.3 GTP, 10 phosphocreatine, pH adjusted to 7.2.

Strategy for studying synaptic versus extrasynaptic NMDARs. To assess the effect of memantine on synaptic and extrasynaptic NMDARs in the same neuron, we used microcultured autaptic neurons. Initially, synaptic responses were induced by short-term depolarizations, stable responses were achieved, and then the effect of drug on EPSCs was assessed. Next, taking advantage of the irreversible effect of (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801), we blocked synaptic NMDARs by repeated synaptic stimulation in the presence of 1–10 μM MK-801. Under these conditions, extrasynaptic NMDARs are spared from blockade (Hardingham et al., 2002; Papadia et al., 2008; Okamoto et al., 2009). Thus, we could then test the effect of memantine on pure extrasynaptic NMDAR-mediated responses induced by bath application of NMDA (100 μM) in conjunction with coagonist glycine (10 μM). Considering peak responses, the relative ratio of the synaptic to extrasynaptic current in these autaptic neurons was 8.4 ± 1.0 ($n = 18$). However, this calculation may underestimate the value for the extrasynaptic current since we did not use saturating concentrations of NMDA. Similarly, the effect of memantine on extrasynaptic responses (vs synaptic responses) may have been underestimated for the same reason since memantine is an uncompetitive antagonist (i.e., at a fixed concentration, the antagonist blocks higher concentrations of NMDA to a relatively greater extent since the action of the antagonist is contingent on previous activation of the receptor by the agonist).

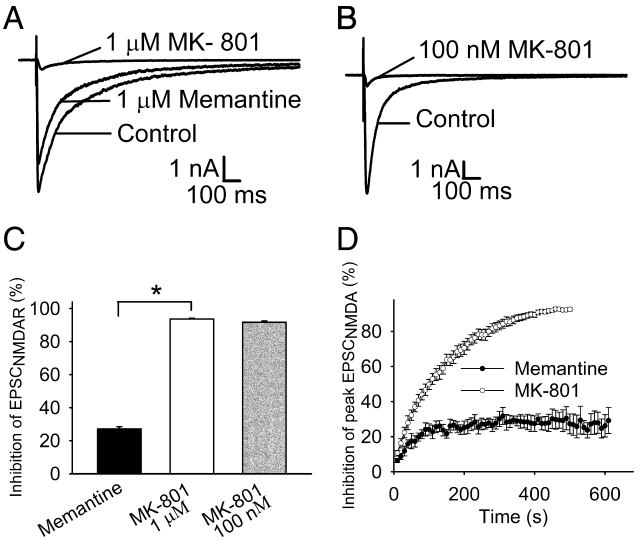

Figure 1. Low micromolar concentrations of memantine manifest relatively little effect on synaptic NMDAR-mediated currents. ***A***, At 1 μM, memantine exerted a minimal effect on the NMDAR component of the EPSC. For comparison, nearly complete blockade by 1 μM MK-801 is shown. ***B***, At 100 nM, MK-801 also nearly completely blocked synaptic NMDARs. ***C***, After the inhibitory effect reached a plateau, 1 μM memantine blocked only 27.1 ± 1 3% of the NMDAR-mediated component of the EPSC, whereas equimolar MK-801 rendered virtually complete blockade (93.6 ± 0.5%). To reach a plateau with 100 nM MK-801, we performed prolonged recordings and still observed 91.6 ± 0.7% blockade of synaptic currents; 10 nM MK-801, although not reaching a plateau, also blocked nearly all of the $EPSC_{NMDAR}$ (data not shown). ***D***, At 1 μM, memantine blockade of the NMDAR-mediated component of the EPSC reached a plateau within 100 s, whereas 1 μM MK-801 required ~500 s. Electrical pulses to elicit EPSCs were delivered every 10 s. Values are mean ± SEM, measured at peak current ($n = 28$; *$p < 0.0001$ by *t* test).

Results

Minimal effect of low micromolar concentrations of memantine on synaptic NMDARs of autaptic neurons

Using the whole-cell configuration of the patch-clamp technique, we recorded the NMDAR component of the EPSC from autapses of solitary hippocampal neurons. The NMDAR component was isolated by suppressing the AMPA receptor-mediated component of the EPSC with 10 μM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione). Depolarization of the neuron to +80 mV for 1 ms from a holding potential of −70 mV evoked robust excitatory autaptic NMDAR-mediated currents. Memantine (1 μM) blocked 27.1 ± 1.3% of the NMDAR component of the EPSC under these conditions after the inhibitory effect had reached a plateau (Fig. 1).

In contrast, previous studies have shown that MK-801 blocks virtually all of the NMDAR-mediated synaptic current after multiple stimuli, reflecting its use-dependent and irreversible characteristics (Huettner and Bean, 1988; Rosenmund et al., 1993; Hardingham et al., 2002; Papadia et al., 2008; Okamoto et al., 2009). In line with these findings, here in the hippocampal autaptic preparation, a similar concentration of MK-801 (1 μM) blocked 93.6 ± 0.5% of this current ($n = 9$; $p < 0.001$). Nonetheless, this might be considered an unfair comparison since the affinity of MK-801 is so much higher (in the low nanomolar range) than that of memantine (low micromolar). Although MK-801 has a much slower off-rate and thus higher apparent affinity for NMDAR-associated channels than memantine, the rate constant for macroscopic blockade (or on-rate) is relatively slow for both drugs (MacDonald et al., 1991; Chen and Lipton, 1997). This fact limits how low a concentration of MK-801 can be as-

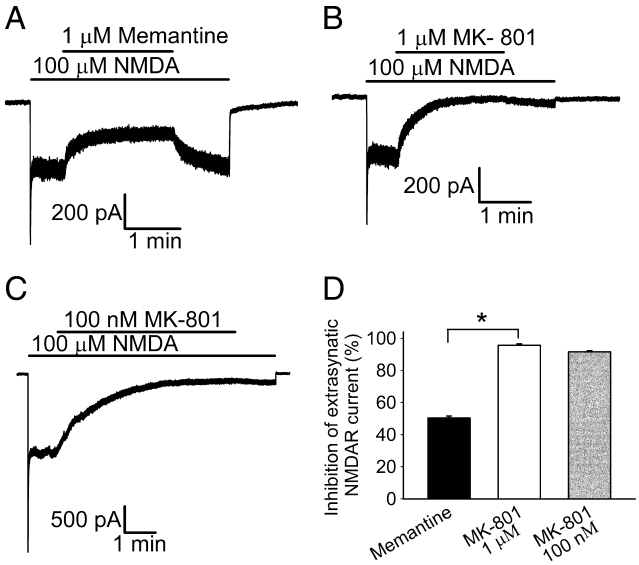

Figure 2. Memantine block of extrasynaptic NMDARs activated by bath application of 100 μM NMDA. ***A***, ***B***, Inhibitory effect of 1 μM memantine or MK-801 on NMDA-induced extrasynaptic currents at a holding potential of −70 mV. ***C***, At 100 nM, MK-801 also completely blocked extrasynaptic NMDARs. ***D***, Memantine blocked predominantly extrasynaptic NMDAR-mediated current (50.4 ± 1.3%) compared with synaptic NMDARs (27.1 ± 1.3%), whereas MK-801 blocked both equally well regardless of concentration (~96% block by 1 μM and ~92% at 100 nM). Values are mean ± SEM ($n = 28$; *$p < 0.0001$).

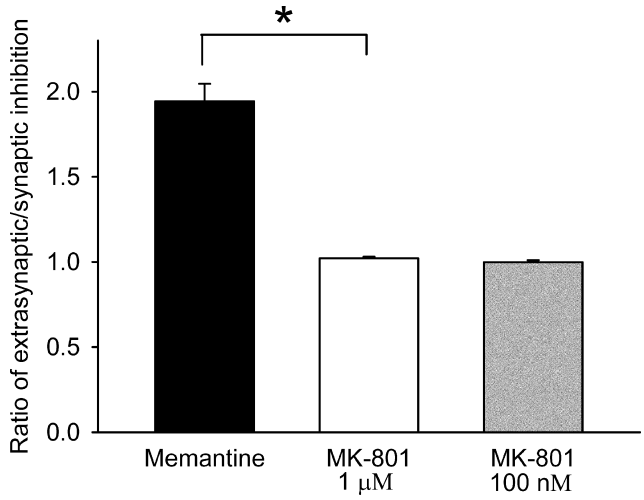

Figure 3. Memantine preferentially blocks extrasynaptic NMDARs. A low (1 μM) concentration of memantine blocked extrasynaptic over synaptic NMDARs at a ratio of ~2:1, whereas 1 μM or 100 nM MK-801 blocked both synaptic and extrasynaptic NMDARs equally. Values are mean ± SEM ($n = 28$; *$p < 0.0001$).

sessed electrophysiologically because of the lengthy time required to reach plateau conditions at very low concentration. When we repeated this experiment with 100 nM MK-801, we still found virtually complete blockade of synaptic responses (Fig. 1*B*,*C*). We also attempted this experiment with 1–10 nM MK-801, but as reported previously (Huettner and Bean, 1988), the on-rate of MK-801 is so slow at these concentrations that, although we still saw substantial block of synaptic responses, the extent of inhibition did not reach a plateau in the course of even long recording sessions lasting several tens of minutes. Importantly, however, compared with low nanomolar concentrations of MK-801, micromolar memantine relatively spared synaptic NMDAR function.

Low micromolar concentrations of memantine block extrasynaptic NMDAR-mediated currents

Since MK-801 almost completely and irreversibly blocks synaptic NMDAR currents under our experimental conditions, we could isolate extrasynaptic NMDAR currents by preapplying MK-801 during repetitive electrical stimulation to activate and subsequently block the autaptic/synaptic currents (see Materials and Methods) (Hardingham et al., 2002; Okamoto et al., 2009). We then tested in the same neuron the effect of memantine on extrasynaptic NMDARs. We activated extrasynaptic NMDARs by bath application of 100 μM NMDA plus 10 μM glycine; 1 μM memantine inhibited approximately one-half of the extrasynaptic NMDAR-mediated current at a holding potential of −70 mV (50.4 ± 1.3%; $n = 24$) (Fig. 2*A*). As a comparison, 1 μM MK-801 blocked 95.7 ± 0.9% of the NMDA-induced extrasynaptic current using the same paradigm, and 100 nM blocked 91.6 ± 0.7% (Fig. 2*B–D*).

Memantine preferentially blocks extrasynaptic over synaptic NMDARs

We compared the synaptic and extrasynaptic effects of NMDAR open-channel blockers on the same autaptic neuron and found that 1 μM memantine blocked extrasynaptic more than synaptic NMDARs by approximately twofold (1.94 ± 0.10; $n = 24$) (Fig. 3). Thus, in the same neuron, low micromolar concentrations of memantine preferentially block extrasynaptic NMDARs, while relatively sparing physiological synaptic function. As a comparison, concentrations of MK-801 of 1 μM or 100 nM showed no preference, blocking both synaptic and extrasynaptic NMDARs approximately equally (1.02 ± 0.01 and 1.00 ± 0.01; $n = 12$) (Fig. 3).

Memantine preferentially blocks extrasynaptic over synaptic NMDARs under pathological conditions

Under normal conditions, at resting membrane potentials, most NMDARs are blocked by extracellular Mg^{2+}, which occupies the channel (Mayer et al., 1984; Nowak et al., 1984; Dingledine et al., 1999; Chen and Lipton, 2006). Under pathological conditions, however, cells continue to depolarize until Mg^{2+} is repelled, and Mg^{2+} block is mostly relieved (Zeevalk and Nicklas, 1992; Chen and Lipton, 2006). It has been reported that memantine blockade is less voltage dependent than Mg^{2+}, so memantine can still block NMDARs effectively under relatively depolarized conditions (Wrighton et al., 2008). Thus, we next tested the effect of memantine on synaptic and extrasynaptic NMDAR-mediated currents in the presence of 1 mM Mg^{2+} at a holding potential of −25 mV, which mimics severe neuropathological conditions. In these experiments with relatively depolarized holding potentials, we tested a concentration of 10 μM memantine because we recently showed that this was the concentration that was effectively present at NMDAR-operated channels in previous rodent studies as well as human clinical trials of the drug under pathological conditions (Chen and Lipton, 1997; Lipton, 2006; Okamoto et al., 2009). In this paradigm, to monitor synaptic currents the neurons received a brief hyperpolarizing prepulse to remove Na^+ channel inactivation and then returned to −25 mV to elicit an EPSC (for details, see Materials and Methods).

Memantine (10 μM) blocked 39.8 ± 2.5% of the NMDAR-mediated component of EPSCs in the presence of 1 mM Mg^{2+}, whereas the same concentration of memantine blocked 64.3 ± 2.5% of the extrasynaptic NMDAR current in the same neuron (Fig. 4). Thus, under pathological conditions, when NMDARs are overactivated and Mg^{2+} is repelled from the channels because

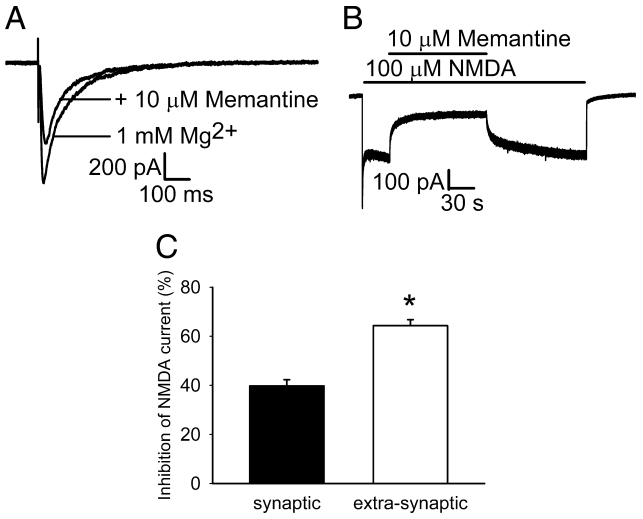

Figure 4. Therapeutic concentrations of memantine block pathological NMDA-induced currents. ***A***, At 10 μM, memantine exerted a minimal effect on the NMDAR-mediated component of the EPSC at a holding potential of -25 mV in the presence of 1 mM extracellular Mg^{2+}. ***B***, Under the same conditions, 10 μM memantine displayed a substantial inhibitory effect on 100 μM NMDA-induced extrasynaptic currents. ***C***, Memantine (10 μM) significantly blocked more extrasynaptic than synaptic NMDAR-mediated currents when neurons were pathologically depolarized ($V_h = -25$ mV; extrasynaptic inhibition, 64.3 $\pm$ 2.5%; synaptic inhibition, 39.8 $\pm$ 2.5%; $n = 7$; *$p < 0.0005$ by paired t test). Values are mean $\pm$ SEM.

of depolarization, memantine still exhibits preferential blockade of extrasynaptic over synaptic NMDAR-mediated currents.

Discussion

Excessive activation of NMDARs by excitatory amino acids such as glutamate may contribute to neuronal damage in a number of neurological disorders (Rothman and Olney, 1986; Choi et al., 1988; Lipton and Rosenberg, 1994). Recently, Hardingham and colleagues have demonstrated that activation of synaptic versus extrasynaptic NMDARs induces opposite effects on various cellular pathways, including CREB (cAMP response element-binding protein)/CBP (CREB-binding protein) transcriptional events that trigger the neuroprotective PGC-1α pathway and antioxidant cascades (Hardingham et al., 2002; Lipton, 2008; Papadia et al., 2008). Physiological stimulation of synaptic NMDARs activates these neuronal survival pathways, whereas excessive stimulation of extrasynaptic NMDARs leads to the loss of mitochondrial membrane potential, inhibition of survival cascades, and activation of cell death pathways. Additionally, we and colleagues have recently shown that neurodegenerative disorders characterized by protein misfolding may also be influenced by electrical activity, with extrasynaptic NMDARs mediating formation of toxic soluble oligomers of aberrant proteins, whereas synaptic activity favors the "walling off" of misfolded proteins into less toxic macroscopic aggregates (Okamoto et al., 2009). Hence, an important strategy for potential therapeutic yet clinically tolerated NMDAR antagonists would be to block excessive extrasynaptic currents while relatively sparing synaptic activity. Such maintenance of normal neurotransmission would also minimize undesirable side effects caused by disrupting physiological activity.

The hippocampal autaptic neuronal preparation afforded us the opportunity to make an unequivocal quantitative comparison of the effect of NMDAR antagonists on synaptic versus extrasynaptic receptors by recording from "the same cell" under identical conditions. Memantine at low micromolar concentrations is clinically tolerated while offering beneficial effects in patients with moderate-to-severe Alzheimer's disease (Wesemann et al., 1980; Hesselink et al., 1999; Danysz et al., 2000; Reisberg et al., 2003; Tariot et al., 2004; Chen and Lipton, 2006; Okamoto et al., 2009). It is thus the only known clinically tolerated NMDAR antagonist. Here, we show that memantine at therapeutic concentrations improves the balance of excitatory activity by preferentially blocking extrasynaptic NMDARs, even under pathologically depolarizing conditions, while relatively sparing synaptic communication. MK-801, which is poorly tolerated clinically (Lipton, 2006, 2007), blocked both synaptic and extrasynaptic NMDARs to the same degree. The fact that low micromolar memantine displays a preference for blocking extrasynaptic over synaptic NMDARs may help explain why memantine manifests a therapeutic effect with few side effects in its clinical use in humans (Lipton, 2006, 2007; Okamoto et al., 2009). That is, memantine relatively spares physiological synaptic transmission important for normal brain function. Extrasynaptic NMDARs, however, which are excessively activated under pathological conditions and harmful to the brain, are effectively antagonized by memantine.

Why does memantine block extrasynaptic more than synaptic NMDARs? The answer to this query could give us hints for future therapeutic drug development, not only for modulation of NMDAR activity but for other targets as well (Lipton, 2006, 2007). There are at least three potential explanations: (1) memantine cannot enter synaptic NMDAR-operated ion channels as readily as those of extrasynaptic NMDARs; (2) synaptic and extrasynaptic NMDARs are composed of different NR2 subunits for which memantine manifests different potencies; (3) disparate activity patterns of synaptic and extrasynaptic NMDAR-operated channels result in a differential degree of memantine blockade. To begin to distinguish between these alternatives, we have applied memantine for long periods of time ($\geq$10 min) to achieve a plateau of the antagonistic effect, and yet we still obtained differential blockade of synaptic and extrasynaptic receptors, excluding the first explanation. It has been reported that synaptic and extrasynaptic NMDARs are composed of different NR2 subunits, with NR2A predominating at synaptic receptors and NR2B at extrasynaptic NMDARs, at least in mature neurons (Dalby and Mody, 2003; Thomas et al., 2006). We also found that this is indeed the case in our autaptic neuronal cultures (data not shown). Memantine, however, was found to have little preference among these NR2 subunits (Bresink et al., 1996). In the presence of 1 mM extracellular Mg^{2+}, 0.1–1 μM memantine was recently shown to exert a more potent effect at NR2C/D subunits than NR2A/B subunits (Kotermanski and Johnson, 2009); however, at 10 μM, the concentration estimated to be present at the channel mouth after therapeutic dosing [Okamoto et al. (2009), their supplemental information], memantine is quite effective at each NR2 subtype (Kotermanski and Johnson, 2009). Our recent result that the specific binding site for memantine is in the channel region of the NR1 subunit (present in all NMDARs) may explain these previous findings (Chen and Lipton, 2005) and makes the second potential explanation very unlikely as the main mechanism for the safety profile of memantine.

Our results are most compatible with the third explanation (i.e., the known disparate activity patterns of synaptic and extrasynaptic NMDARs result in differential memantine blockade). We previously proposed that, because of its uncompetitive nature and relatively rapid unblocking or "off-rate" from NMDAR-associated channels, memantine predominantly acts as an open-channel blocker in the presence of prolonged elevation of glutamate concentration or prolonged channel activity (on the scale of tens of minutes or longer), as seen for extrasynaptic receptors, but is

relatively inactive when glutamate is elevated for only milliseconds, as in synaptic transmission (Lipton, 2006, 2007; Wyllie and Chen, 2007; Okamoto et al., 2009). Heretofore, however, it has been difficult to observe the action of known concentrations of memantine at neuronal synapses because of their compact geometry. This experiment was necessary to confirm the fact that synaptic channels are blocked to a lesser extent than extrasynaptic channels by memantine; indeed, we accomplished that objective in the present experiments. Here, we show unequivocally for the first time that memantine indeed preferentially blocks extrasynaptic receptors over synaptic receptors, which may contribute to the clinical tolerability of memantine. Hence, future drug development must attend to the differential blockade of extrasynaptic versus synaptic NMDAR-mediated currents with the goal of achieving a higher ratio of blockade of extrasynaptic/synaptic current, which should lead to even better tolerability and neuroprotective efficacy.

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