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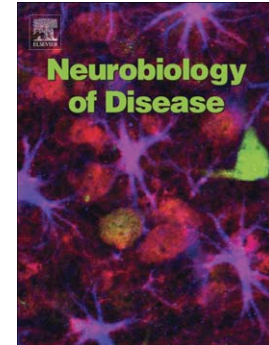
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Effects of memantine on the excitation-inhibition balance in prefrontal cortex

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Abbreviations:

AD, Alzheimer's disease

NMDAR, *N*-methyl-D-aspartate receptor

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

E/I, excitation/inhibition

EGFP, enhanced green fluorescent protein

PFC, prefrontal cortex

sEPSCs, spontaneous excitatory postsynaptic currents

sIPSCs, spontaneous inhibitory postsynaptic currents

mIPSC, miniature inhibitory postsynaptic currents

dIPSCs, disynaptic inhibitory postsynaptic currents

moEPSCs, monosynaptic excitatory postsynaptic currents

PV, parvalbumin

TTX, tetrodotoxin

NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline

ACSF, artificial cerebrospinal fluid

Abstract

Memantine is one of the few drugs currently approved for treatment of Alzheimer's disease (AD). The clinical effects of memantine are thought to be associated with inhibition of NMDA receptors (NMDARs). Surprisingly, other open-channel NMDAR blockers have unacceptable side effects that prevent their consideration for AD treatment. One of the mechanisms proposed to explain the therapeutic benefits of memantine involves preferential decrease of excitatory drive to inhibitory neurons in the cortical circuitry and consequent changes in balance between excitation and inhibition (E/I). In this study we addressed effects of memantine on E/I balance in the prefrontal cortex (PFC). We found that a moderate concentration of memantine shifted E/I balance away from inhibition in the PFC circuitry. Indeed, memantine decreased the frequency and amplitude of spontaneous inhibitory postsynaptic currents in pyramidal neurons while leaving spontaneous excitatory postsynaptic currents unaffected. These circuitry effects of memantine were occluded by the competitive NMDAR inhibitor AP-5, and thus are associated with NMDAR inhibition. We also found that memantine decreased feed-forward disynaptic inhibitory input to pyramidal neurons, which is thought to be mediated by parvalbumin (PV)-positive interneurons. Accordingly, memantine caused a greater decrease of the amplitude of NMDAR-mediated synaptic responses in PV-positive interneurons than in pyramidal neurons. Finally, memantine reduced firing activity in PV-positive interneurons while increasing firing in pyramidal neurons. This study elucidates a novel mechanism of action of memantine associated with shifting of the E/I balance away from inhibition in neocortical circuitry, and provides important insights for AD drug development.

Keywords

Alzheimer's disease

Memantine

NMDA receptors

Prefrontal cortex

Inhibition

Excitation/inhibition balance

Whole-cell recording

Channel block

Introduction

Alzheimer's disease (AD) is a devastating brain disorder that heavily burdens the aging American population. Although a daunting challenge, creating new AD therapeutics can be aided by better understanding of the mechanisms of action of existing AD drugs.

Memantine has been used to treat AD and other dementias for more than two decades. Although memantine binds to a number of receptors, it is generally believed that the therapeutic effects of memantine are mainly associated with *N*-methyl-D-aspartate receptor (NMDAR) channel block (Lipton, 2006, Parsons et al., 2007, Johnson et al., 2015). There is no general agreement on how NMDAR channel block by memantine slows cognitive decline in AD patients. Several mechanisms of memantine action have been proposed, the majority of which are based on memantine's neuroprotective action. The neuroprotective effect of memantine could result from prevention of excitotoxicity due to excessive NMDAR excitation in pathological brain conditions (Lipton, 2006), or from preferential inhibition of extrasynaptic NMDARs (Leveille et al., 2008, Xia et al., 2010) based on the hypothesis that extrasynaptic NMDAR activation can lead to cell death (Hardingham and Bading, 2010) (but see (Wroge et al., 2012)). However, there is evidence arguing against therapeutic effects of memantine based solely on neuroprotection, e.g. the lack of beneficial effects in early-stage AD, and the rapidity of its effects (Johnson and Kotermanski, 2006).

The ability of memantine to slow cognitive decline in AD patients has also been proposed to result from partial correction of an AD-induced alteration of cortical excitation/inhibition (E/I) balance (Schmitt, 2005). A delicate balance of excitatory and inhibitory elements in the cortex is essential to circuit function, and disturbances of this balance can lead to pathological conditions (Homayoun and Moghaddam, 2007, Haider and McCormick, 2009). There is strong evidence

that the E/I balance is shifted away from excitation in AD. A decrease in cortical activity (Rombouts et al., 2000) and PFC hypometabolism in the prefrontal cortex (PFC) (Schroeter et al., 2012) were reported in AD patients. Data from AD postmortem brains indicate that, whereas excitatory pyramidal neurons in the PFC are prone to neurodegeneration (Hof and Morrison, 2004), PFC inhibitory neurons that express the Ca^{2+} binding protein parvalbumin (PV) are spared (Hof et al., 1991). In transgenic AD model mice, despite substantial neuronal loss in the PFC, no changes in PV-positive and calretinin-positive interneurons were detected (Lemmens et al., 2011). There is substantial loss of cortical spines, the principal site of excitatory synaptic input onto pyramidal neurons, in both the neocortex and hippocampus of AD patients (Cochran et al., 2014). Note, however, that some data do not support decreased excitation in AD. For instance, in the parietal cortex and hippocampus of mice expressing human amyloid precursor protein, nonconvulsive seizure activity resulting from an aberrant increase in network excitability was detected (Palop et al., 2007), and increased excitability of hippocampal CA1 pyramidal neurons was detected in APP/PS1 AD model mice (Siskova et al., 2014).

In this study we explore the hypothesis that memantine can shift the E/I balance in cortical circuitry away from inhibition (Johnson and Kotermanski, 2006) by preferentially reducing NMDAR-mediated excitation of inhibitory neurons. This hypothesis is based on two observations: (1) in physiological Mg^{2+} , memantine at therapeutic concentrations preferentially inhibits NMDARs that contain the GluN2C or GluN2D subunits (Kotermanski and Johnson, 2009); (2) in adult cortex and hippocampus, the GluN2D subunit is preferentially expressed in inhibitory neurons (Monyer et al., 1994, Standaert et al., 1996). If this hypothesis is correct, then memantine at a concentration that preferentially inhibits GluN2C and GluN2D-containing NMDARs should be more effective at reducing the action potential frequency of inhibitory

neurons than of excitatory neurons. To test this prediction we explored in mouse PFC the effects of 10 μ M memantine on inhibitory and excitatory synaptic inputs to pyramidal neurons, on NMDAR-mediated synaptic inputs to PV-positive interneurons, and on synaptically-activated action potentials in pyramidal neurons and PV-positive interneurons.

Materials and Methods

Experiments were performed on PFC slices from 3-7 month old CB6-Tg(Gad1-EGFP)G42jh/J mice of either sex, which express EGFP in PV-positive interneurons (<http://jaxmice.jax.org/strain/007677.html>). All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and approved by our Institutional Animal Care and Use Committee. Mice were deeply anesthetized with chloral hydrate and decapitated. The brain was quickly removed and immersed in ice-cold pre-oxygenated artificial cerebrospinal fluid (ACSF). Coronal slices containing PFC were made as previously described (Povysheva et al., 2006). Throughout experiments, ACSF (31-32°C, perfused with a 95% O₂/5% CO₂) of the following composition was used (in mM): 126 NaCl, 2.5 or 10 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 2 CaCl₂, 24 NaHCO₃, 10-20 glucose; pH~7.3.

Electrophysiological recordings

Whole-cell recordings were performed from layer 2-3 neurons visualized by IR-DIC videomicroscopy as previously described (Povysheva and Johnson, 2012). Pyramidal neurons were identified by their apical dendrites and triangular somata. Interneurons were recognized based on EGFP green fluorescence. Patch electrodes (5-10 MΩ open-tip resistance) were filled with a solution containing (in mM): 105 Cs-gluconate, 2 MgCl₂, 10 NaCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP, and 10 BAPTA; pH 7.25. Alexa hydrazide 568 (0.075%; Molecular Probes, Eugene, OR) was added to the solution for morphological identification of the recorded neurons. Voltage and current recordings were performed with a Multi-Clamp 700A amplifier (Axon Instruments, Union City, CA). Signals were filtered at 2 kHz and acquired at a sampling rate of 10 kHz using Clampex 10.2 software (Molecular Devices Corporation,

Sunnyvale, CA). Access resistance typically was 10-20 M Ω and remained relatively stable during experiments ($\leq 30\%$ increase). Corrections were made for liquid junction potential (-13 mV). We used: gabazine (10 μ M) to inhibit GABA_A receptors; 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX; 20 μ M) to inhibit kainate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors); D-2-amino-5-phosphopentanoic acid (AP-5; 50 μ M) to inhibit NMDARs; tetrodotoxin (TTX; 0.5 μ M) to inhibit voltage-gated Na⁺ channels; memantine (10 μ M). Gabazine, NBQX, and AP-5 were purchased from Ascent Scientific LTD (Bristol, UK); TTX and memantine from Sigma (St. Louis, MO).

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded at +12 mV, spontaneous excitatory post-synaptic currents (sEPSCs) were recorded at -70 mV, both in ACSF with elevated K⁺ (10 mM). Miniature inhibitory postsynaptic currents (mIPSCs) were recorded at +12 mV in TTX. We found that sIPSCs and sEPSCs were stable in the absence of memantine for the typical duration of experiments. A bipolar stimulating electrode placed on the border of white matter and layer 6 (Povysheva et al., 2006) was used to evoke NMDAR EPSCs, sequences of feed-forward monosynaptic and disynaptic IPSCs (**Fig. 4**), and trains of EPSPs and synaptically-activated action potentials (**Fig. 5**). NMDAR EPSCs were recorded at -70 mV, and feed-forward monosynaptic and disynaptic IPSCs were recorded at -50 mV, both in elevated K⁺. Trains of EPSPs and synaptically-activated action potentials were evoked by five stimuli delivered at 25 Hz in elevated K⁺; 14 - 18 trains with an intertrain interval of 10 sec were used for each cell in each condition.

Electrophysiological, morphological, and statistical data analysis

Membrane properties and morphology of neurons were analyzed as previously described (Povysheva et al., 2006). Spontaneous and miniature events were analyzed using the

MiniAnalysis Program (Synaptosoft, Decatur, GA) as previously described (Povysheva and Johnson, 2012). Amplitude of evoked synaptic responses was measured on averaged traces as the most positive (for IPSC) or the most negative (for EPSC) current value compared to baseline current using Clampfit. Charge per sIPSC or sEPSC was the averaged area above or below mean baseline current; mean current was mean charge per spontaneous current multiplied by spontaneous current frequency. Spike probability for each stimulus number in a train was calculated as the total number of stimulation-evoked spikes divided by the total number of stimuli across all stimulus trains given to a cell in each condition. Two-tailed paired t-test was used for paired comparisons and ANOVA for correlated samples followed by Tukey post hoc test was used for group comparisons using Excel (Microsoft Corp., Redmond, WA). Values are presented as mean \pm SEM.

Results

Memantine reduces inhibition of pyramidal neurons

First we assessed memantine's effects on inhibitory input to pyramidal neurons, the cell type that is the main source of excitation in PFC circuitry. All pyramidal neurons studied exhibited typical physiological phenotypes including large action potential amplitude, strong adaptation (**Fig. 1A, left**), and typical morphology (triangular cell body, pronounced apical dendrite, etc.; **Fig. 1A, right**). To isolate sIPSCs, pyramidal neurons were held at +12 mV (Cossart et al., 2001), a voltage far from the reversal potential of GABA_A receptor responses, but close to that of glutamatergic responses, which were undetectable at +12 mV: all visible events were abolished by gabazine (**Fig. 1B**). The concentration of external K⁺ was elevated to 10 mM to depolarize neurons, increase their firing rate (McBain, 1995) and, consequently, increase sIPSC frequency (control, 6.1±0.5 Hz; elevated K⁺: 8.2±0.6 Hz; n=11, p<0.01). Blockade of neuronal firing with TTX reduced both average amplitude (control, 27.1±2.7 pA; TTX, 15.6±1.5 pA; n=8, p<0.01) and frequency (control, 7.8±0.6 Hz; TTX, 5.4±0.5 Hz; p<0.001) of sIPSCs.

If 10 μM memantine preferentially inhibits NMDAR-mediated excitation of inhibitory neurons, then memantine should cause a greater reduction of action potential frequency in inhibitory neurons than in excitatory neurons. As a result, memantine should preferentially reduce the frequency of action potential-dependent IPSCs recorded in pyramidal neurons. Since sIPSCs include both action potential-dependent IPSCs and smaller action potential-independent miniature IPSCs, we predict that memantine should reduce both the frequency and amplitude of sIPSCs. To test this prediction we evaluated sIPSC amplitude, frequency, and mean current before, and 10 min after application of memantine, when memantine's effects had reached steady

state. We used a memantine concentration of 10 μM , which, although above the likely therapeutic brain concentration range ($\sim 0.5\text{--}1\text{ }\mu\text{M}$ (Parsons et al., 2007)), is a concentration that allowed reliable quantification of memantine's effects in slice experiments. Critically, based on previous measurements using recombinant receptors (Kotermanski and Johnson, 2009), 10 μM is a memantine that inhibits GluN1/2C and GluN1/2D receptors ($\sim 85\%$ reduction) more effectively than GluN1/2A or GluN1/2B receptors (44–49% reduction) in physiological Mg^{2+} . Through paired comparisons, memantine was found to decrease inhibitory input to pyramidal neurons by reducing sIPSC amplitude, frequency (**Fig. 1C–E**), and mean current (control, $3.1 \pm 0.33\text{ pA}$; memantine, $2.3 \pm 0.31\text{ pA}$; $n=11$, $p<0.05$; see **Fig. 2H**).

We next determined whether this disinhibitory action of memantine was associated specifically with blockade of NMDARs. We observed that bath application of the NMDAR antagonist AP-5 reduced the amplitude and frequency of sIPSCs ($n=5$, $p<0.001$), and subsequent application of memantine did not have any additional effect on sIPSCs in pyramidal neurons ($p>0.1$); **Fig. 1F,G**. Occlusion by AP-5 confirms that the disinhibitory effect of memantine results predominantly from inhibition of NMDARs.

Memantine does not change excitation of pyramidal neurons

To shift the E/I balance, memantine would need to have differential effects on inhibitory and excitatory drive. Thus, next we assessed the effects of memantine on sEPSCs, which were used to assess excitatory drive onto pyramidal neurons. The concentration of external K^+ again was elevated to 10 mM to increase neuronal firing rate. To isolate sEPSCs, pyramidal neurons were held at -70 mV , a voltage far from the reversal potential of excitatory responses, but close to that of inhibitory responses ($E_{\text{Cl}^-} = -59\text{ mV}$), which were undetectable at -70 mV : all visible events were abolished by NBQX (**Fig. 2A**). In addition, NMDARs were almost fully inhibited by Mg^{2+}

at -70 mV, and thus did not contribute significantly to sEPSC amplitude. Paired comparison of sEPSCs in pyramidal neurons before and after memantine application did not reveal any effects of the drug on sEPSC amplitude, frequency (**Fig. 2B-D**), or mean current (control, 0.63 ± 0.13 pA; memantine, 0.75 ± 0.15 pA; $n=10$; **Fig. 2H**).

Interestingly, a near-saturating concentration of AP-5 decreased amplitude and frequency of sEPSC (**Fig. 2E-G**). Thus, whereas a moderate concentration of memantine decreases inhibitory inputs without affecting excitatory inputs, shifting E/I balance away from inhibition, AP-5 decreases both excitatory and inhibitory drive. These results suggest that inhibition of a subset of NMDARs by 10 μ M memantine results in circuitry effects qualitatively distinct from those produced by full NMDAR inhibition (**Fig. 2H**).

Memantine does not affect mIPSCs

Memantine could reduce sIPSC frequency through at least two general mechanisms: by reducing firing of inhibitory neurons, resulting in a decrease of spike-dependent sIPSC frequency, or by reducing GABA release as a result of inhibiting presynaptic NMDARs activated by ambient glutamate (Duguid and Smart, 2004). To test the latter mechanism we blocked neuronal firing with TTX and continuously recorded whole-cell current at +12 mV. Frequency and amplitude of mIPSCs were evaluated before, and 10 min after bath application of memantine. Memantine did not affect pyramidal neuron mIPSC amplitude or frequency (**Fig. 3**), indicating that the effects of memantine on sIPSCs are not associated with a reduction of mIPSCs. Thus, memantine does not affect action potential-independent mIPSCs, indicating that memantine most likely reduces inhibitory drive to pyramidal neurons by decreasing action potential-driven inhibitory responses.

Role of PV-positive interneurons in memantine's effects

Since PV-positive interneurons provide strong inhibitory control of pyramidal neuron activity (Wilson et al., 2012), reduction of PV-positive interneuron activity by memantine could contribute to pyramidal neuron disinhibition. Therefore we next we examined the effects of 10 μ M memantine on PV-positive interneurons. First, we asked whether memantine preferentially reduces NMDAR-mediated synaptic responses of PV-positive interneurons. PV-positive interneurons expressing EGFP were identified in CB6-Tg(Gad1-EGFP)G42Zjh/J mice (**Fig. 4A, left**). All cells that expressed EGFP possessed a typical fast-spiking phenotype (**Fig. 4A, right**) (Markram et al., 2004). NMDAR-mediated postsynaptic currents in PV-positive interneurons were evoked by extracellular stimulation in the presence of NBQX and gabazine. These responses were abolished by AP-5. 10 μ M memantine reduced the amplitude of NMDAR EPSCs in PV-positive interneurons by almost 50% (control, 43 ± 6 pA; memantine, 22 ± 4 pA; $n=7$, $p<0.01$; **Fig. 4B,C**). Interestingly, the effect of memantine on NMDAR EPSC amplitude was substantially less in pyramidal neurons, where memantine decreased amplitude only by 22% (control, 30 ± 3 pA; memantine, 23 ± 3 pA; $n=7$, $p<0.01$; **Fig. 4B,C**). This differential effect of memantine on NMDAR-mediated responses in excitatory and inhibitory neurons may contribute to the effect of memantine on cortical E/I balance.

Fast-spiking PV-positive interneurons ensure temporal fidelity of pyramidal neuron outputs through feed-forward disynaptic inhibition (Povysheva et al., 2006, Cruikshank et al., 2010). If memantine decreases excitability of PV-positive interneurons, disynaptic inhibitory input to pyramidal neurons should be weakened. To test this prediction, disynaptic inhibitory postsynaptic currents (dIPSCs) were evoked in pyramidal neurons by extracellular stimulation in elevated K^+ to increase neuronal excitability. A holding voltage of -50 mV was used for these

experiments to permit recording of both monosynaptic EPSCs (moEPSCs) and dIPSCs. Importantly, dIPSCs (and EPSCs) were abolished by addition of AMPAR and NMDAR antagonists, confirming that dIPSCs were disynaptic and were not contaminated by monosynaptic IPSCs (**Fig. 4D**). The dIPSC starts before the moEPSC peak (latency of isolated dIPSC (**Fig. 4F**; see below), 4.07 ± 0.21 ms; moEPSC time-to-peak (**Fig. 4D**), 8.02 ± 0.35 ms, $n=6$, $p<0.001$), and thus reduces the moEPSC peak amplitude. As a result, a memantine-induced decrease in dIPSC amplitude could “unmask” and appear to increase moEPSC amplitude. Indeed, application of memantine increased the measured peak amplitude of moEPSCs (control, 76 ± 20 pA; memantine, 116 ± 30 pA; $n=5$, $p<0.05$; **Fig. 4D,E**).

The overlap in the time course of dIPSCs and moEPSCs (Povysheva et al., 2006) prevented accurate measurement of dIPSC latency or peak amplitude at -50 mV. To measure dIPSC onset and changes in amplitude produced by memantine, we depolarized the pyramidal neurons to -8 mV, a voltage at which moEPSCs were not detectable (**Fig. 4F,G**) (Povysheva et al., 2006). Importantly, any residual moEPSC would be inward at -8 mV, ensuring that we would not misinterpret a memantine-induced decrease of moEPSC amplitude as a decrease of dIPSC amplitude. At -8 mV dIPSC onset was preceded by a flat baseline (**Fig. 4F,G**), allowing reliable measurement of latency as well as amplitude. dIPSC latency (4.07 ± 0.21 ms, $n=6$) was over twice as long as that of moEPSC measured at more negative potentials (1.9 ± 0.09 ms; $n=5$, $p<0.001$), supporting the disynaptic nature of dIPSCs. Application of memantine reduced the amplitude of dIPSCs measured at -8 mV (control, 114 ± 22 pA; memantine, 63 ± 17 pA; $n=6$, $p<0.001$; **Fig. 4G,H**), providing further evidence that preferential inhibition of NMDARs on PV-positive interneurons contributes to the disinhibitory effects of memantine.

Memantine effects on spiking activity in pyramidal neurons and PV-positive interneurons

Next, we directly assessed the effects of memantine on firing activity of pyramidal neurons and PV-positive interneurons. We used trains of extracellular stimuli of near-threshold intensities to produce EPSPs and action potentials in each cell type. In accord with our hypothesis, memantine decreased firing activity in PV-positive interneurons (**Fig. 5A**), while increasing firing in pyramidal neurons (**Fig. 5B**).

Next we analyzed spike probability in pyramidal neurons and PV-positive interneurons separately for each of the 5 stimuli in the train. Importantly, we found that memantine substantially reduced spike probability in responses to the stimulus 1 in PV-positive interneurons, but not in pyramidal neurons (**Fig. 5C**). Unlike responses to the first stimulus, responses to stimuli 2-5 could be influenced by activation of excitatory and inhibitory neurons by previous stimuli, and thus assess effects of cortical circuitry on spike probability. For stimuli 2-5 memantine showed a tendency to increase spike probability for each stimulus in pyramidal neurons, but to decrease spike probability in PV-positive interneurons. These effects were significant for stimulus 2 in pyramidal neurons and for stimuli 2 and 3 in PV-positive interneurons.

Thus, 10 μ M memantine had opposite effects on stimulus-evoked firing of excitatory pyramidal neurons and inhibitory PV-positive interneurons. These results are in accord with data in Figs. 1, 2, and 4 indicating that memantine preferentially decreases excitation of inhibitory interneurons. Our results support the hypothesis that a moderate concentration of memantine shifts the E/I balance away from inhibition.

Discussion

In this study we unveiled a mechanism of action of memantine associated with a change in the E/I balance in PFC circuitry. By measuring excitatory and inhibitory responses in PFC pyramidal neurons, we found that memantine shifts the E/I balance away from inhibition. The effects of memantine on the NMDAR-mediated synaptic responses, disynaptic activation, and synaptically evoked spiking of PV-positive inhibitory neurons are consistent with memantine's disinhibitory effects. The hypothesized mechanism by which memantine-mediated disinhibition provides modest clinical benefits to AD patients is summarized in **Fig. 6**.

Memantine action on NMDARs reduces cortical inhibition

It remains unresolved how inhibition by memantine of NMDARs, which are expressed at lower than normal levels in AD (Jacob et al., 2007), can produce clinical benefits for AD patients. Here we present evidence that memantine causes cortical disinhibition by decreasing inhibition of excitatory neurons, which could partially compensate for an E/I imbalance in cortical circuitry in AD (**Fig. 6**). We found that memantine reduced inhibitory drive to pyramidal neurons (**Fig. 1**) but did not affect excitatory drive (**Fig. 2**). The reduction of inhibitory drive by memantine was occluded by the NMDAR antagonist AP-5 (**Fig. 1**), indicating that NMDAR inhibition mediates memantine's disinhibitory effects. Other NMDAR channel blocking drugs, including ketamine, phencyclidine, and MK-801 also have been shown to mediate cortical disinhibition (Farber, 2003, Homayoun and Moghaddam, 2007).

There are at least two plausible general mechanisms by which memantine inhibition of NMDARs could disinhibit pyramidal neurons: memantine could inhibit presynaptic NMDARs at GABAergic synapses onto pyramidal neurons, or could reduce the frequency of spikes generated

by inhibitory neurons. There is evidence that presynaptic NMDAR activation at GABAergic synapses can mediate an increase in mIPSC frequency (Duguid and Smart, 2004). Here we showed that memantine had no effect on mIPSCs (**Fig. 3**), arguing that memantine inhibition of presynaptic NMDARs on GABAergic terminals is not responsible for pyramidal neuron disinhibition. Thus, the disinhibitory action of memantine is likely to be associated with reduction of action potential generation by inhibitory neurons. Interestingly, based on the reduction of event frequency by TTX (Results), ~70% of sIPSCs are mIPSCs. The moderate decrease of sIPSC frequency by memantine (**Fig. 1D**) therefore probably represents a substantial decrease of spike-dependent sIPSCs.

PV-positive interneurons as a target of memantine

Our data suggest that PV-positive interneurons, which synapse close to the spike generation site on pyramidal neurons and strongly influence pyramidal neuron activity, are involved in memantine action. We demonstrated that in pyramidal neurons, memantine reduced the amplitude of dIPSCs (**Fig. 4G,H**). This reduction suggests that memantine may decrease activity of fast-spiking PV-positive interneurons (Povysheva et al., 2006). We also found that memantine reduced evoked NMDAR-mediated synaptic responses more effectively in PV-positive interneurons than in pyramidal neurons (**Fig. 4B,C**), again indicating that memantine induces disinhibition. Importantly, we found that memantine reduced synaptically-evoked firing activity in PV-positive interneurons while increasing firing in pyramidal neurons (**Fig. 5**).

In addition to PV-positive interneurons, other interneuron subpopulations are likely to be involved in disinhibition by memantine; in contrast to our results, NMDAR-mediated synaptic responses have been found to play a relatively small role in excitation of PV-positive interneurons (Rotaru et al., 2011). However, preferential inhibition by memantine of NMDAR

responses is not a general property of interneurons: in hippocampus, memantine was found to inhibit synaptic NMDAR responses of likely cholecystinin interneurons less potently than pyramidal neuron responses (Martina et al., 2013).

Preferential inhibition of NMDAR responses in PV-positive interneurons could result from their expression of the GluN2D NMDAR subunit (Monyer et al., 1994, Standaert et al., 1996) coupled with memantine's selective inhibition of GluN1/2C and GluN1/2D receptors in physiological Mg^{2+} (Kotermanski and Johnson, 2009). However, the NMDAR-mediated synaptic response deactivation time course we observed (**Fig. 3B**) is inconsistent with GluN1/2C or GluN1/2D receptor kinetics. A possible explanation is that PV-positive interneurons express GluN2D-containing triheteromeric NMDARs, which, like GluN1/2D receptors, appear to be weakly inhibited by Mg^{2+} (Huang and Gibb, 2014); the deactivation kinetics of GluN2D-containing triheteromeric receptors have not been characterized. Alternatively, memantine could reduce PV-positive interneuron spiking by acting on GluN2C- or GluN2D-containing extrasynaptic receptors, or on presynaptic NMDARs at excitatory synapses onto PV-positive interneurons.

In AD patients, the extensive loss of neocortical excitatory synapses coupled with relative sparing of PV-positive interneurons may lead to a pathological E/I imbalance. We propose that one mechanism by which memantine produces clinical benefits is partial compensation for this E/I imbalance through reduction of interneuron activity (**Fig. 6**).

Acknowledgments

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Figure legends

Figure 1. Memantine reduced inhibition of PFC pyramidal neurons. **A.** Identification of pyramidal neurons. Left, responses produced in pyramidal neurons by depolarizing and hyperpolarizing current pulses; right, pyramidal neuron filled with fluorescent dye during recording and later confocally reconstructed (scale bar 20 μm). **B-G.** sIPSCs recorded in pyramidal neurons at a holding voltage (V_{hold}) of +12 mV in elevated (10 mM) K^+ . **B.** 10 μM gabazine eliminated spontaneous events, indicating that sEPSCs were not visible at +12 mV. **C.** sIPSCs recorded before and after 10 μM memantine application. **D,E.** Memantine decreased sIPSC amplitude (D) and frequency (E). Control (Con), black; memantine (Mem), gray; $n=11$. **F,G.** Effects of memantine were occluded by 50 μM AP-5. AP-5 decreased sIPSC amplitude (left, $p<0.001$) and frequency (right, $p<0.01$); subsequent application of AP-5 + memantine had no additional effect on amplitude or frequency ($p>0.1$); ANOVA followed by Tukey post-hoc test.

Figure 2. Memantine did not affect excitation of PFC pyramidal neurons. **A-G.** sEPSCs recorded in pyramidal neurons at -70 mV in elevated K^+ . **A.** 20 μM NBQX eliminated spontaneous events, indicating that sIPSCs were not visible at -70 mV. **B.** sEPSCs recorded before and after memantine application. **C,D.** Memantine did not change sEPSC amplitude (C) or frequency (D). Control, black; memantine, gray; $n=10$. **E.** sEPSCs recorded before and after application of 50 μM AP-5. **F-G.** AP-5 decreased amplitude (F) and frequency (G) of sEPSCs. Control, black; AP-5, gray; $n=8$. **H.** Comparison of changes in mean inhibitory (I) and excitatory (E) currents. Control, black; AP-5, light gray; memantine, dark gray.

Figure 3. Memantine did not affect mIPSCs in pyramidal neurons. **A.** mIPSCs recorded in TTX at +12 mV before and after memantine application. **B,C.** Memantine did not change amplitude (**B**) or frequency (**C**) of mIPSCs in pyramidal neurons. Control, black; memantine, grey; n=6.

Figure 4. Memantine reduced disynaptic inhibition in PFC pyramidal neurons. **A.** Left, live image of PV-positive interneurons expressing EGFP (arrows) in a PFC slice (scale bar 50 μ m). Right, responses produced in PV-positive interneurons by depolarizing and hyperpolarizing current pulses recorded in ACSF. **B,C.** Evoked NMDAR-mediated EPSCs recorded in ACSF with NBQX and gabazine at -70 mV were reduced in amplitude by memantine more substantially in PV-positive interneurons (n=7) than in pyramidal neurons (n=7). Memantine did not change the decay time (tau of a single-exponential fit, $p>0.1$) of NMDAR EPSCs in either cell type. **D,E.** moEPSCs and dIPSC were evoked in pyramidal neurons in elevated K^+ at -50 mV. The measured peak amplitude of the moEPSC was increased by memantine, probably as a result of reduction of the overlapping dIPSC (see **F**). The moEPSC-dIPSC sequence was abolished by NBQX and AP-5 (**D**). **F.** Series of superimposed dIPSCs were recorded in elevated K^+ at -8 mV. **G,H.** dIPSC amplitude was reduced by memantine. The dIPSC was abolished by NBQX + AP-5 (**G**). **B,D,G,** averaged traces (n=8-10).

Figure 5. Memantine affects spiking activity in pyramidal neurons and PV-positive interneurons. **A,B.** The probability of action potential generation in response to trains of 5 stimuli was decreased by memantine in PV-positive interneurons (**A**), but increased in pyramidal neurons (**B**). **C.** The effect of memantine on the probability of action potential generation in response to each stimulus in the 5-stimulus trains (\$, $p<0.001$; &, $p<0.05$).

Figure 6. Partial compensation for E/I balance in AD pathology by memantine.

Hypothesized mechanism by which memantine-induced shift of the E/I balance away from inhibition in cortical circuitry produces clinical benefits for AD patients. In normal cortex there is a balance between excitatory (Exc) and inhibitory (Inh) synaptic activity (left). One consequence of AD pathology is decreased cortical excitation (middle). We found that application of memantine decreases synaptic inhibitory input to pyramidal cells by reducing NMDAR-mediated excitation of inhibitory neurons, which, we hypothesize, partially compensates for AD pathology-associated reduction of excitatory drive (right; smaller diameter inhibitory axons represent lower firing frequency of inhibitory neurons).

Figure 1 (Povysheva & Johnson)

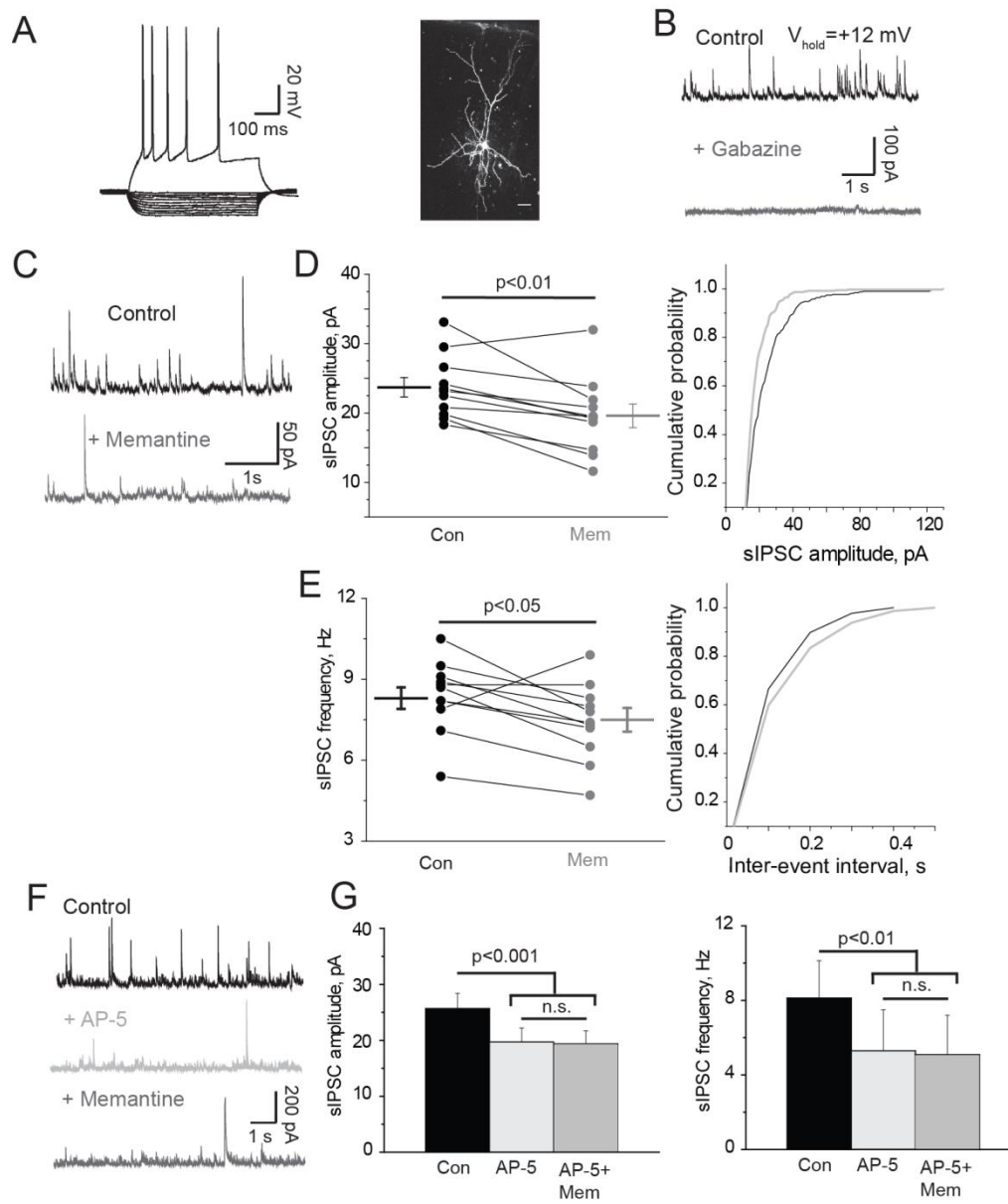


Figure 2 (Povyshева & Johnson)

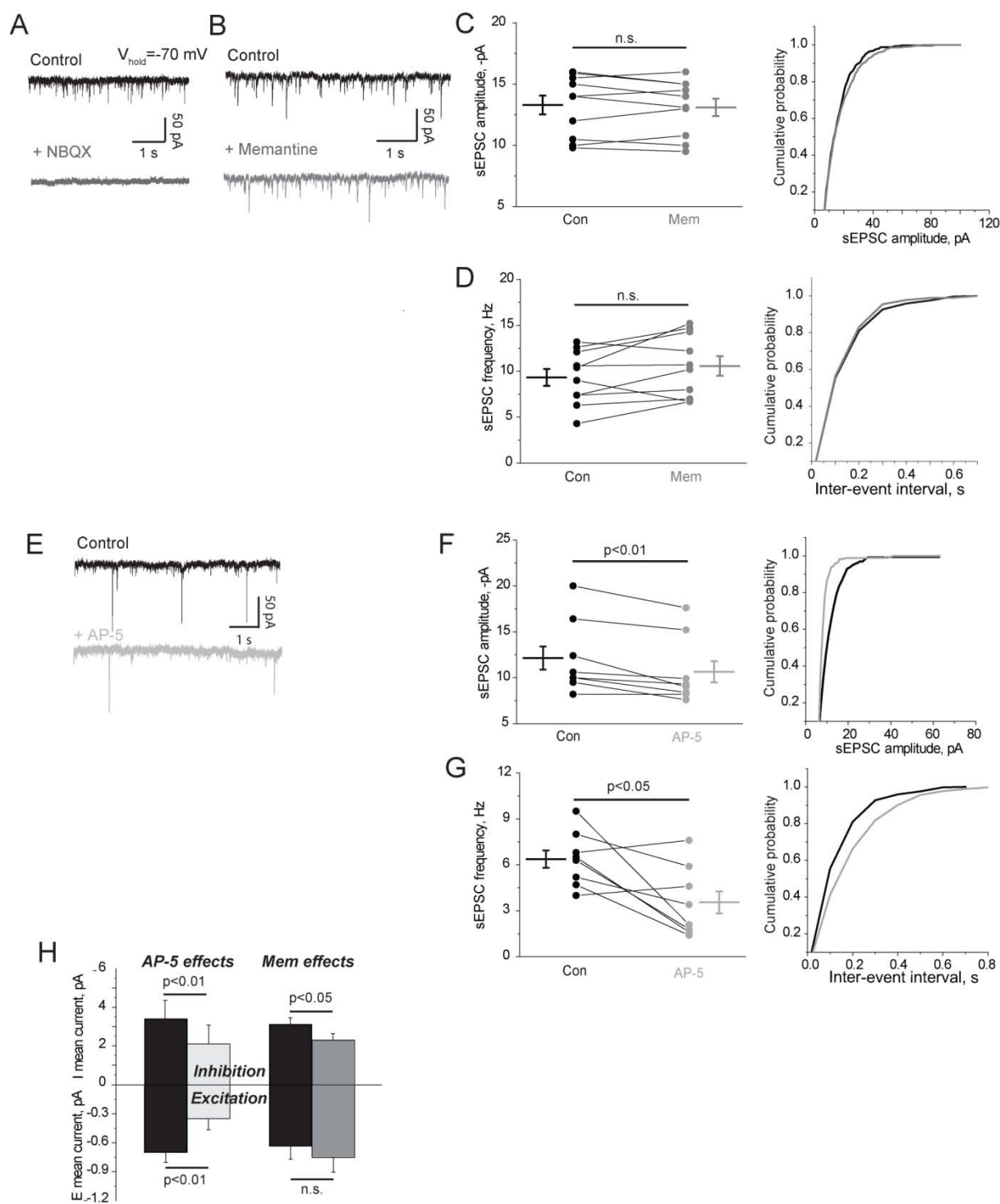


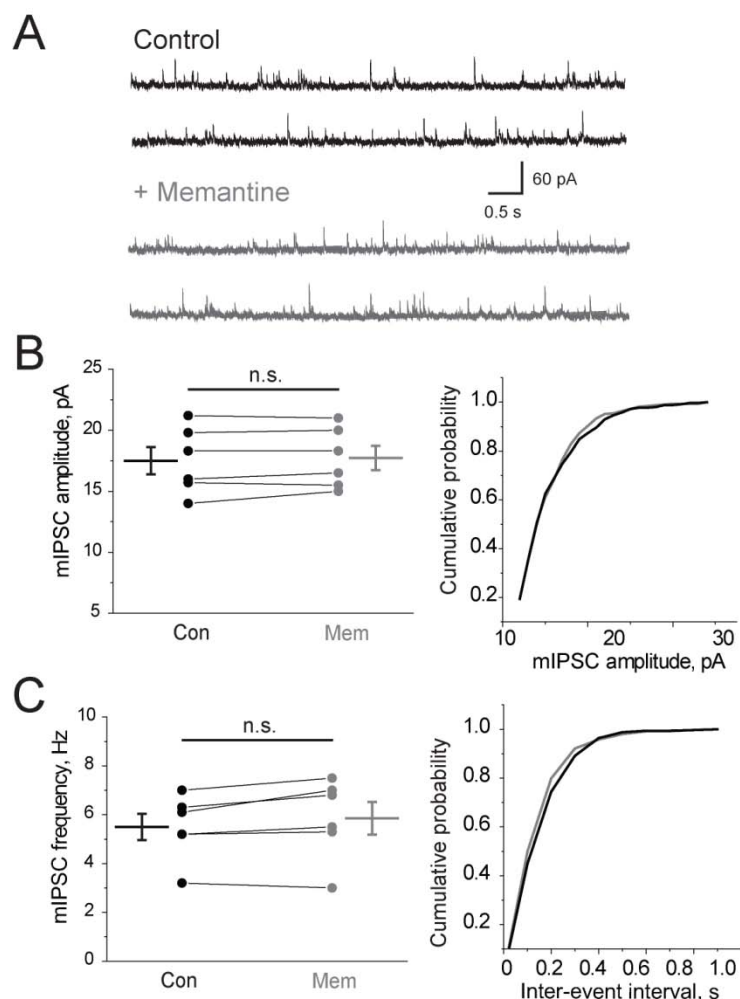
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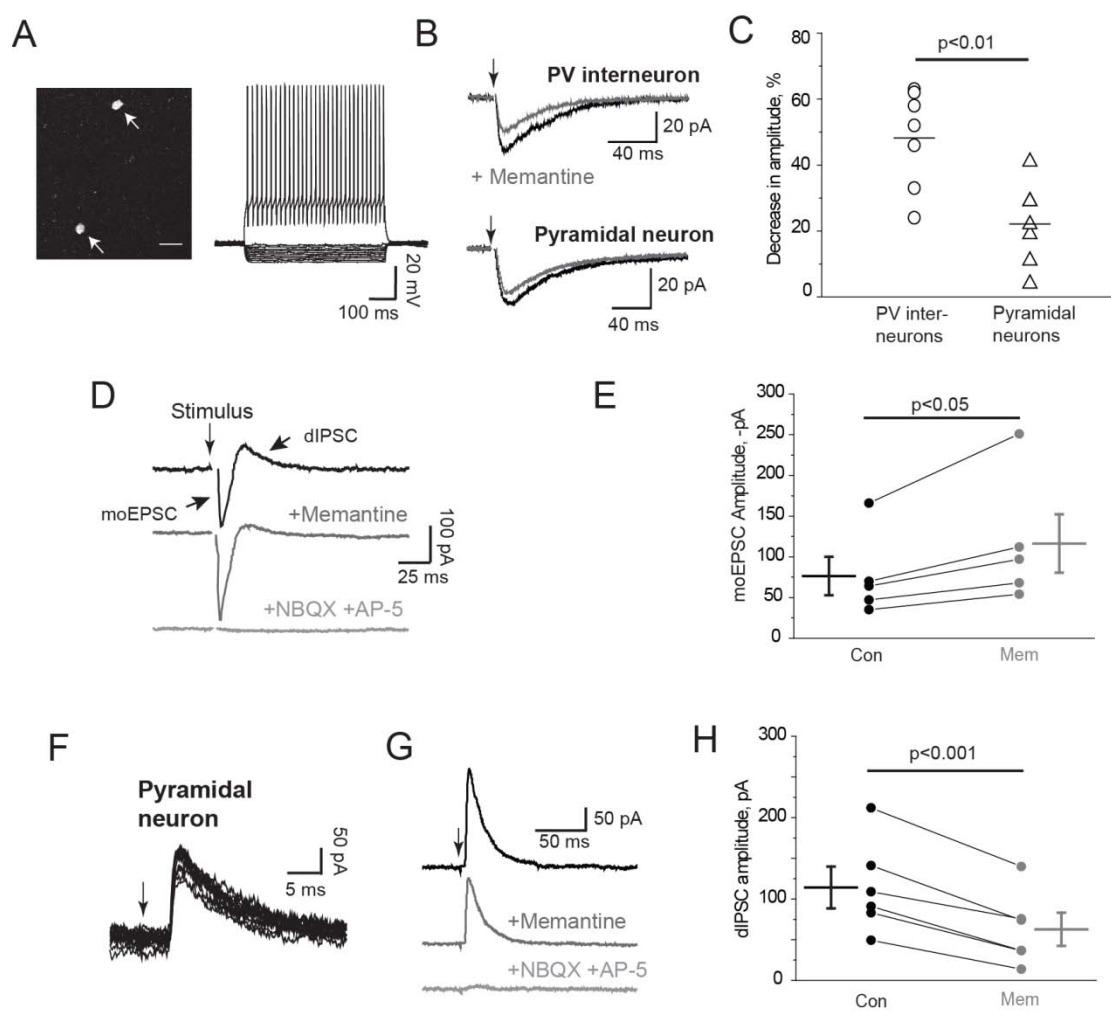
Figure 4 (Povysheva & Johnson)

Figure 5 (Povysheva & Johnson)

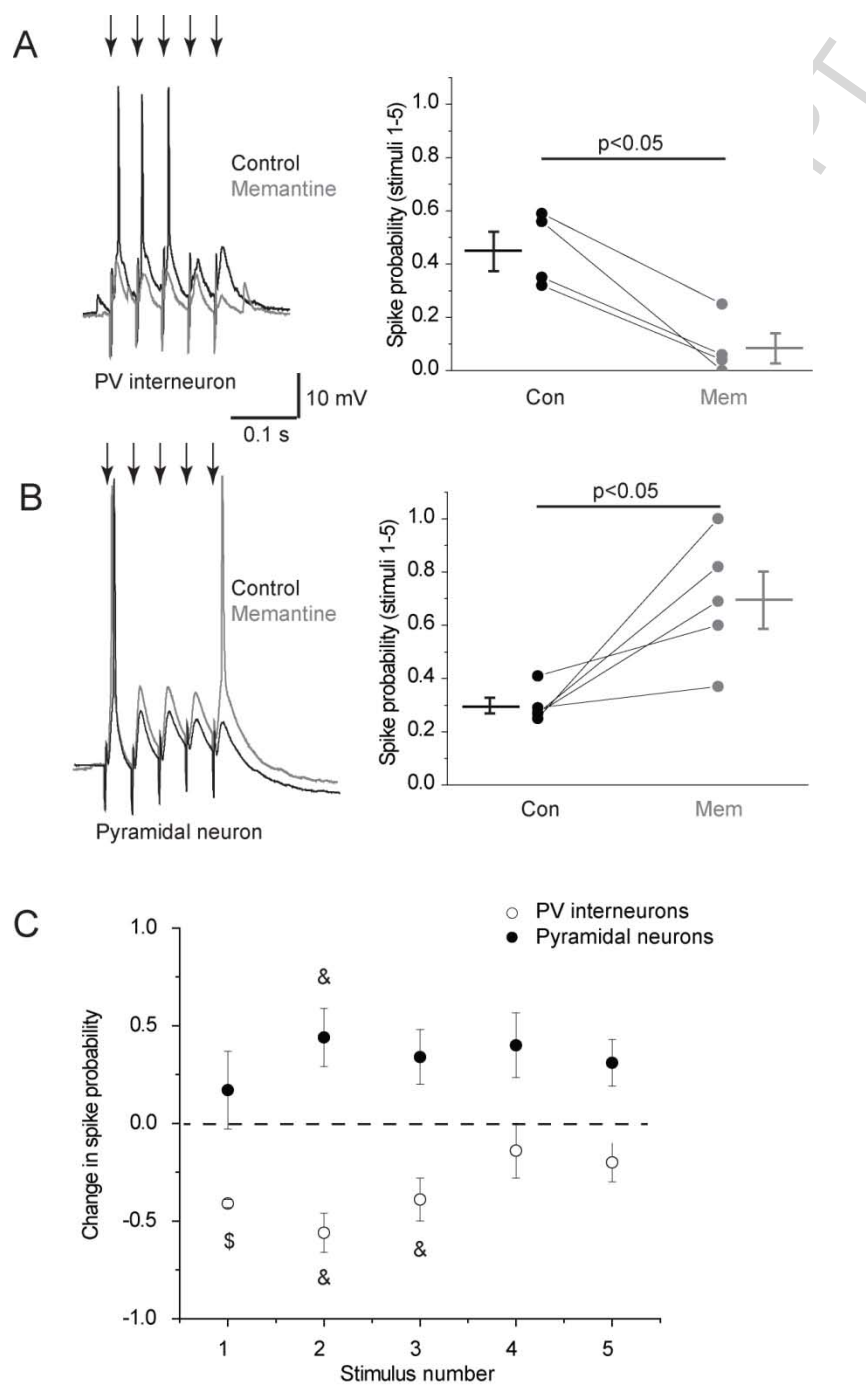
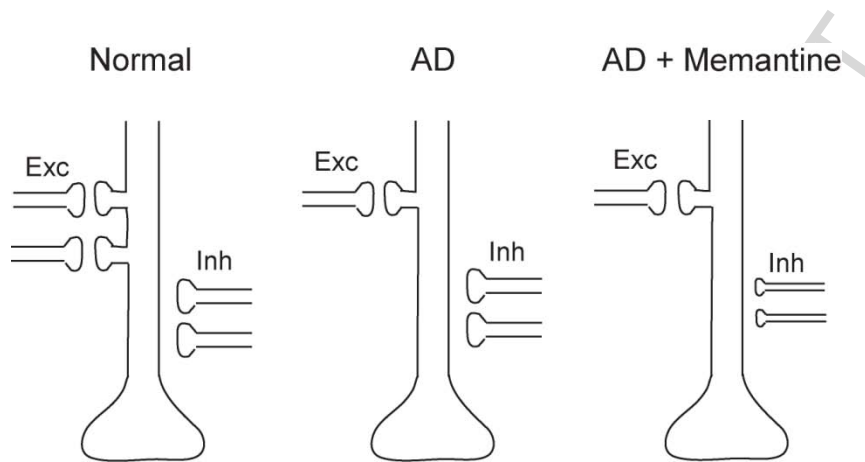


Figure 6 (Povysheva & Johnson)

Highlights

- Novel actions of the Alzheimer's Disease (AD) drug memantine were investigated
- Memantine disinhibits cortex by shifting excitation/inhibition balance
- Memantine reduces NMDAR responses in PV+ interneurons more than in pyramidal neurons
- Memantine reduces spiking in PV+ interneurons, increases spiking in pyramidal neurons
- Disinhibition may be an effective mechanism of action for new AD therapeutics