Activation of Brain Histaminergic Neurotransmission: A Mechanism for Cognitive Effects of Memantine in Alzheimer’s Disease

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ABSTRACT

We previously reported that some N-methyl-D-aspartate (NMDA)-receptor antagonists enhanced histamine neuron activity in rodents. Here, we have investigated the effects of memantine, an NMDA-receptor antagonist used for the treatment of Alzheimer’s disease, on histaminergic neurotransmission. In vitro, memantine antagonized native NMDA receptors with a micromolar potency but had no effect at recombinant human histamine receptors. In vivo, a single administration of memantine increased histamine neuron activity, as shown by the 60% increase of tele-methylhistamine (t-MeHA) levels observed in the brain of mice. This increase occurred with an ED₅₀ of 0.3 ± 0.1 mg/kg, similar to that found on inhibition of ex vivo [³H]dizocilpine maleate (MK-801) binding (1.8 ± 1.3 mg/kg). Two days after pretreatment of mice with memantine at 5 mg/kg twice daily for 5 days, t-MeHA levels were enhanced by 50 ± 7% (p < 0.001), indicating a long-lasting activation of histamine neurons. Quantitative polymerase chain reaction analysis was used to explore genes involved in this persistent effect. H₃ receptor mRNAs were strongly increased, but the density of H₃ receptor binding sites was increased solely in hypothalamus (by 141 ± 24%). Up-regulations of brain-derived neurotrophic factor and NMDA-receptor 1 subunit mRNAs were also found but were restricted to hippocampus. mRNA expression of α7-nicotinic receptors remained unchanged in any region. Considering the well established cognitive effects of histamine neurons, the increase in brain t-MeHA levels after single or repeated administration of therapeutic doses of memantine suggests that the drug exerts its beneficial effects on cognitive deficits of Alzheimer’s disease, at least partly, by activating histamine neurons.

Introduction

Histamine neurons constitute a divergent system that arises from the tuberomammillary nucleus in the posterior hypothalamus and projects in a diffuse manner to many cerebral areas. When released from these neurons, histamine triggers its effects in the brain by activating H₁-receptor (H₁R), H₂-receptor (H₂R), and H₃-receptor (H₃R) subtypes, which are all G protein-coupled receptor subtypes. The H₂ receptor (H₂R), which is mainly expressed on immune and hematopoietic cells, may also play a role in brain, because it was recently revealed to be expressed in central neurons (Strakhova et al., 2009). Histamine is then metabolized via transmethylation into tele-methylhistamine (t-MeHA) catalyzed by histamine N-methyltransferase (Brown et al., 2001).

The arousing and cognitive effects of histamine are now largely documented. They are mainly mediated by H₁Rs and occur either directly via excitation of neocortical pyramidal neurons and thalamic relay neurons or indirectly via excitation of ascending cholinergic neurons (Haas and Panula, 2003; Haas et al., 2008). Enhancing histaminergic neurotransmission therefore improves cognition and facilitates various forms of learning (Passani et al., 2004). H₃R inverse agonists, which enhance histamine neuron activity (Morisset et al., 2000a), improve cognition and learning in behavioral studies in rodents (Passani et al., 2004). In addition, clinical data from numerous pharmaceutical companies suggest that they should improve cognitive deficits encountered in Alzheimer’s disease (AD). Despite a strong neurofibrillary degeneration of the tuberomammillary nucleus in AD, our recent
findings show that histaminergic neurons can still be activated in the disease, confirming that they constitute an adequate target to improve the cognitive disorders associated to AD (Motawaj et al., 2010).

Memantine is an orally active drug widely used in the world for the treatment of moderate to severe AD since its recent approval by the European Union and the U.S. Food and Drug Administration. Randomized controlled trials and their meta-analysis have confirmed its beneficial effects to slow the long-term progression of the disease when used alone or in association with cholinesterase inhibitors (Tariot et al., 2004; Winblad et al., 2007; Lopez et al., 2009). These effects result from neuroprotection induced by blockade of glutamatergic NMDA receptors (NMDARs). It is now generally accepted that, in contrast to other NMDAR antagonists, memantine provides neuroprotection while displaying minimal adverse effects, because of its moderate affinity, noncompetitive antagonism, strong voltage dependence, and fast kinetics at NMDARs (Johnson and Kotermanski, 2006; Lipston, 2006; Parsons et al., 2007).

However, memantine also exhibits surprising beneficial effects on cognitive deficits of AD, the mechanisms of which remain poorly understood (Parsons et al., 1999; Schmitt, 2005; Johnson and Kotermanski, 2006; Lipston, 2006; Parsons et al., 2007). We have previously reported that systemic administration of the open-channel blockers phencyclidine and MK-801 enhanced t-MeHA levels (i.e., histamine neuron activity) in mouse brain by 50 to 60% (Faucard et al., 2006). Considering the cognitive effects of histamine, these data suggested that memantine could improve cognitive deficits of AD at least partly by activating histaminergic neurotransmission.

In the present study, we have explored the interaction of memantine with the histaminergic system. We first studied the in vitro profile of memantine at native NMDARs and recombinant human histaminergic receptors. We then investigated the in vivo activity of memantine on histamine neuron activity, by measuring t-MeHA levels, a reliable index of this activity, in the brain of mice after single or repeated administrations.

Materials and Methods

Recombinant Human Histaminergic Receptors. The potency of memantine at the four histaminergic receptors was investigated using HEK-293 cells expressing the human H1 (hH1R), H2 (hH2R), H3 (hH3R), or H4 (hH4R) receptors [HEK(hHxR) cells].

For H3Rs, memantine at increasing concentrations was added in the presence of 3 to 5 nM [3H]mepyramine to membranes from HEK(hH3R) cells. Incubations were run and stopped as described in the section describing [3H]mepyramine binding.

For H2Rs, cAMP accumulation was measured with a LANCE cAMP detection kit (PerkinElmer Life and Analytical Sciences, Waltham, MA). HEK(hH2R) were harvested in Hank’s balanced salt solutions/HEPES buffer, pH 7.4, containing 1 mM 3-isobutyl-1-methylnxantine and 1 mg/ml bovine serum albumin. After centrifugation (1000g for 5 min), the cells (2 x 10^6 per ml) were resuspended in the same buffer. The Alexa Fluor 647-anti cAMP antibody solution (1 μl) was added to the cell suspension (100 μl), and 6-μl aliquots of this mix were dispensed in white microtiter plates (OptiPlate; PerkinElmer Life and Analytical Sciences). The cells were then incubated with 6-μl aliquots of memantine alone or together with 1 μM histamine. After a 1-h incubation at room temperature in the dark, the lysis buffer (0.35% Triton X-100, 10 mM CaCl2, and 50 mM HEPES) containing LANCE Eu-W8044-labeled streptavidin and biotinylated cAMP was added to the cells. After a 2-h incubation at room temperature in the dark, plates were kept overnight at 4°C and read on an EnVision microplate reader (PerkinElmer Life and Analytical Sciences).

For H2Rs, binding assays were performed as described previously (Ligneau et al., 1994; Gbahou et al., 2006). Aliquots of membrane suspensions from HEK(hH2R) cells (~200 μg of protein) were incubated for 60 min at 25°C with 50 pM [3H]idoproyfan alone or together with memantine at increasing concentrations in phosphate buffer (50 mM Na2HPO4/KH2PO4, pH 7.5) (final volume, 200 μl). The nonspecific binding was determined using imetit (1 μM).

On H3R-mediated inhibition of cAMP accumulation, the effects of memantine were studied using HEK-293 cells expressing either the human [HEK(hH3R)] or the rat [HEK(rH3R)] receptor. The cells were incubated with memantine at increasing concentrations in the presence of forskolin (final concentration, 0.5 μM), and cAMP accumulation was measured as described above for H2 receptors.

For H2Rs, binding assays were performed as described previously (Gbahou et al., 2006). Aliquots of membrane suspensions from HEK(hH2R) cells (~200 μg of protein) were incubated for 60 min at 25°C with 3 nM [3H]histamine alone or together with memantine at increasing concentrations in Tris buffer (50 mM Tris-HCl, pH 7.5) (final volume, 200 μl). The nonspecific binding was determined using 5-chloro-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole (JNJ 7777120; 1 μM).

Animals. Male Swiss mice (20–25 g) and male Wistar rats (150–200 g) (Jaunier, Le Genest-Saint-Isle, France) were housed with free access to food and water in a room maintained at 21 to 22°C under a 12-h light/dark cycle with lights on from 7:00 AM.

Intracellular Ca^2+ in Single Cultured Neurons. Primary neuronal cultures from rat cerebral cortex were performed as described previously (Burban et al., 2010). The cerebral cortex was removed from 18-day-old rat embryos. Cells were dissociated with a fire-narrowed Pasteur pipette and were plated on glass slides previously coated with poly L-ornithine (15 μg/ml) and laminin (2 μg/ml) at a seeding density of 10^6 cells/ml. After removal of the coating solution, cells were seeded in neurobasal medium supplemented with B27 (1:50), 2 mM GlutaMAX-I, 5 IU/ml penicillin, and 5 μg/ml streptomycin. In these conditions, cultures of neurons were favored at the expense of glial cells. Neurons were maintained for 7 to 11 days in vitro without medium change at 37°C in a humidified atmosphere containing 5% CO2.

For measurements of cytosolic free Ca^{2+}, cells were loaded for 45 min at 37°C with the calcium dye Fluo-4, acetoxymethyl ester (5 μM Fluo4Bios; Interchim, Montluçon, France) in dye-loading buffer (10 mM HEPES, 137 mM NaCl, 1.2 mM MgCl2, 1.2 mM CaCl2, 0.4 mM NaN3PO4, 6 mM KCl, 10 mM glucose, 1 mg/ml bovine serum albumin, and 0.1% Pluronic acid, pH 7.4). After washing and centrifugation at 1000g for 5 min, the cells were resuspended in magnesium-free dye-loading buffer. Aliquots of the cell suspension were dispensed in 96-well plates and the basal fluorescence intensity (F_{basal}) was determined in a fluorometric plate reader Mithras LB640 (Berthold Technologies, Bad Wildbad, Germany). The required drug solutions were then injected and the fluorescence (F) induced by intracellular calcium (Ca^{2+}) mobilization was measured over a 2-min period. The maximal fluorescence intensity (F_{max}) was determined by subsequent addition of Triton (0.1%) to the cells. The induced Ca^{2+} mobilization was expressed, at the peak of fluorescence, as the percentage of F_{max} over basal fluorescence according to the relationship: [Ca^{2+}]_{i} mobilization = ([F - F_{basal}]/F_{max} - F_{basal}) * 100.

Results were expressed as a percentage of the Ca^{2+} mobilization elicited by 50 μM NMDA.

In Vitro [3H]MK-801 Binding. Hippocampal membranes from adult rats were prepared and extensively washed by four successive centrifugations in 5 mM Tris-HCl buffer, pH 7.4. After the last centrifugation, aliquots of the membrane suspension (~200 μg of protein) were incubated for 15 min at 25°C in 0.5 ml of the same...
medium with 2 nM [3H]MK-801 and, when required, memantine at various concentrations. Incubations were stopped by addition of ice-cold buffer and rapid filtration over vacuum onto GF/B filters (Milipore, Billerica, MA). Specific binding was defined as that inhibited by 1 μM MK-801. Radioactivity present on the filters was determined by liquid scintillation spectrometry.

In other sets of experiments, mice received saline or memantine (5 mg/kg) twice daily for 5 days and were sacrificed after a 2-day washout. Tissues from various mouse brain regions were immediately placed in at least 10 volumes of RNA later RNA Stabilization Reagent (QIAGEN, GmbH, Hilden, Germany). RNAs were extracted using the RNeasy mini kit (QIAGEN) and treated with the RNase-free DNase set (QIAGEN).

**Reverse Transcription.** cDNAs were synthesized from total RNAs by reverse transcription using an Omniscript RT kit (QIAGEN). In brief, 1 μg of total RNA was incubated for 5 min at 65°C and then chilled on ice for 2 min. Reverse transcription buffer (1X), 0.5 mM dNTPs, 1 μM anchored oligo-dt (Thermo Fisher Scientific, Epsom, Surrey, UK), 10 units of Nasin Ribonuclease inhibitor (Promega, Madison, WI), and 4 units of Omniscript reverse transcriptase (QIAGEN) were then added. The 20-μl reaction was incubated for 60 min at 37°C followed by a final 5-min incubation at 94°C for termination.

**Quantitative Real-Time PCR.** Quantitative real-time PCR (qPCR) was performed using a sequence detection system (Prism 7000; Applied Biosystems, Foster City, CA). All reactions were run in a 20-μl reaction volume using 25 ng of cDNA, 1× TaqMan Universal PCR Master Mix without AmpErase uracil N-glycosylase (Applied Biosystems), and 1× TaqMan Gene Expression Assay-on-Demand primers and probes (Applied Biosystems). Primer/probe sets used are listed in Table 1. qPCR parameters were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. Data were collected at 60°C. All data were normalized to β-actin content and expressed as percentages of control sample. Relative quantification was calculated using the 2−ΔΔCt method. For quantification of H3R mRNA expression, the amplified 83-base pair fragment was located at boundaries of exons 2 and 3 of the mouse H3R and was therefore common to all functional isoforms (Rouleau et al., 2004).

**Real-Time PCR.** cDNAs were amplified using Taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR primers used were as follows: histamine H2R: forward, 5′-TGG AAG CTT TGG AGT GTG GTG-3′; reverse, 5′-CAT CTT CAT GCG CTT CTC CA-3′; β-actin: forward, 5′-TGT TGA TGG TGG GAA TGG GTG-3′; reverse, 5′-ACG CAC GAT TCC CCT CTC AG-3′. Reactions were processed using an initial denaturation cycle at 94°C for 5 min, then 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The H2R primers described above were based on the

**TABLE 1**

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<th>Amplicon Length</th>
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<td>92</td>
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</table>

bp, base pair(s).
sequence of the second transmembrane domain and the third intracellular loop. The partial coding sequence amplified corresponded to nucleotides 319 to 1017 of the mouse H₃ receptor and contained the region in which deletions differing in length generate the various functional isoforms of the receptor (Rouleau et al., 2004).

Analysis of Data. The total curves were analyzed with an iterative least-squares method by nonlinear regression using a one-site cooperative model (Gbahou et al., 2006). The method provided estimates for IC₅₀ values, ED₅₀ values, and their S.E.M. The apparent affinity constant (Kᵰ) of memantine evaluated from its inhibition of [³H]MK-801 binding was calculated from its IC₅₀ value, assuming a competitive antagonism and by using the relationship (Cheng and Prusoff, 1973): Kᵰ = IC₅₀/(1 + [S]/Kₚ), where S represents the concentration (2 nM) and Kₚ, the apparent dissociation constant of [³H]MK-801 evaluated under the same conditions at the NMDA receptor (10 ± 2 nM, not shown). Statistical analysis of the data were performed by one-way ANOVA followed by Newman-Keuls test, except for the effects of repeated administration of memantine on t-MeHA levels. In that case, a parametric analysis by two-way ANOVA with pretreatment and/or treatment as an independent variable, followed by a Fisher least significant difference post hoc test, was performed to compare t-MeHA levels between the different groups. Protein contents were determined (Lowry et al., 1951) using bovine serum albumin as the standard.

Radiochemicals and Drugs. Memantine was obtained from Tocris (Bristol, UK). Ciproxifan (CPX) was synthesized by W. Schuette (Freie Universität, Berlin, Germany). MK-801, histamine, NMDA, triprolidine, imetit, and tele-methylhistamine were from Sigma-Aldrich (St Louis, MO). [¹²⁵I]Iodoproxyfan (2200 Ci/mmol), [³H]mepyramine (25.8 Ci/mmol), [³H]histamine (52 Ci/mmol), and [³H]MK-801 (27.5 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences.

Results

Effects of Memantine at Recombinant Histamine Receptors. In binding assays, memantine at concentrations up to 100 µM did not affect specific [³H]mepyramine binding to membranes from HEK(hH₃R) cells, specific [¹²⁵I]iodoproxyfan binding to membranes from HEK(hH₄R) cells, or specific [³H]histamine binding to membranes from HEK(hH₂R) cells (not shown).

In cAMP assays, histamine enhanced cAMP formation in HEK(hH₂R) cells with an EC₅₀ value of 0.42 ± 0.22 µM and a maximal cAMP formation of ~300 nM (final concentration) reached at 10 µM histamine (not shown). Memantine at concentrations up to 100 µM had no effect alone and did not inhibit the effect of 1 µM histamine, indicating no agonist or antagonist property of the drug at H₂Rs. As expected from cells displaying a high level of constitutive activity of the H₂R, the reference compound thioperamide behaved as an inverse agonist and strongly enhanced (by ~600%) cAMP formation in HEK(hH₂R) and HEK(hH₄R) cells, whereas memantine had no effect at concentrations up to 100 µM (not shown).

Effects of Memantine at Native NMDA Receptors. Variations of intracellular Ca²⁺ were measured in cultured neurons from rat cerebral cortex loaded with the fluorescent probe Fura-4 acetoxyethyl ester and incubated with the drug-containing magnesium-free buffer (Fig. 1). NMDA (50 µM) markedly increased intracellular Ca²⁺ in all the cultures tested. Memantine inhibited the effect of NMDA in a concentration-dependent manner and with an IC₅₀ value of 24 ± 8 µM (Fig. 1).

The interaction of memantine with NMDARs in vitro was also studied on the nonequilibrium [³H]MK-801 binding to extensively washed membranes from adult rat hippocampus. Memantine inhibited [³H]MK-801 binding completely and in a concentration-dependent manner. Analysis of its inhibition curve by nonlinear regression led to an apparent Kᵰ value of 0.4 ± 0.1 µM with a pseudo-Hill coefficient of 0.85 ± 0.08 (Fig. 2A). The ex vivo [³H]MK-801 binding assay is useful to measure the brain penetration and in vivo receptor occupancy of ligands acting on the NMDAR complex. After intraperitoneal administration, memantine inhibited ex vivo [³H]MK-801 binding with an ED₅₀ of 1.8 ± 1.3 mg/kg (Fig. 2B). Its maximal inhibitory effect (~35 ± 3%) (Fig. 2B) was lower than that of MK-801 itself (~80%, not shown).

Effects of a Single Administration of Memantine on tele-Methylhistamine Levels in Mouse Brain. A single intraperitoneal administration of the H₃R inverse agonist CPX (3 mg/kg) or NMDA receptor antagonist MK-801 (0.3 mg/kg), used as controls, enhanced t-MeHA levels by 81 ± 6

![Fig. 1. Effect of memantine on NMDA-induced increase in intracellular Ca²⁺ in cultured neurons from rat cerebral cortex. Results are expressed as percentages of the increase induced by NMDA (50 µM). Values are means ± S.E.M. of five to nine determinations from three separate experiments. *p < 0.05; **p < 0.01; ***p < 0.001 versus NMDA (one-way ANOVA, Student-Newman-Keuls post hoc test).](image1)

![Fig. 2. Effect of memantine on [³H]MK-801 binding measured in vitro to rat hippocampal membranes (A) and ex vivo to mouse brain membranes (B). [³H]MK-801 (2 nM) was incubated for 15 min at 25°C with the membranes. Memantine was added in vitro at increasing concentrations (A) or administered intraperitoneally to mice at increasing doses 30 min before sacrifice (B). Data are means ± S.E.M. from four separate experiments with quadruplicate determinations.](image2)
and 37 ± 4%, respectively (250 ± 8 and 189 ± 5 versus 138 ± 4 ng/g, respectively, p < 0.001). The single intraperitoneal administration of memantine increased t-MeHA levels in a dose-dependent manner (Fig. 3), with an ED₅₀ value of 0.3 ± 0.1 mg/kg and a maximal effect of ~60% reached at 5 mg/kg (Fig. 3).

**Effects of Repeated Administrations of Memantine on t-Methylhistamine Levels in Mouse Brain.** Mice received intraperitoneal saline or memantine (at 5 mg/kg), twice daily for 5 days, and after either a 2-day (Fig. 4A) or 7-day (Fig. 4B) interruption (washout), changes in t-MeHA levels were evaluated 90 min after intraperitoneal administration of saline or memantine (0.3 or 5 mg/kg). After a 2-day washout, two-way ANOVA indicated that memantine significantly changed t-MeHA levels (F₅.₇₅ = 25.46; p < 0.001). Post hoc analysis revealed that after pretreatment with saline, treatment with memantine at 0.3 and 5 mg/kg significantly increased t-MeHA levels observed in controls (saline) by 29 ± 5 and 68 ± 6%, respectively (174 ± 7 and 227 ± 8 versus 135 ± 6 ng/g, respectively) (Fig. 4A).

After a 2-day washout, pretreatment with memantine (5 mg/kg), significantly increased basal levels in controls (saline) by 50 ± 7% (203 ± 9 versus 135 ± 6 ng/g, p < 0.001) (Fig. 4A). These basal levels were further significantly increased by 21 ± 12 and 45 ± 10% after treatment with memantine at 0.3 or 5 mg/kg, respectively (231 ± 14 and 263 ± 10 versus 203 ± 9 ng/g, respectively) (Fig. 4A).

After a 7-day washout, two-way ANOVA indicated that memantine significantly changed t-MeHA levels (F₅.₇₉ = 38.39 p < 0.001). Pretreatment with memantine no longer significantly increased control t-MeHA levels (saline) (148 ± 5 versus 135 ± 6 ng/g, p = 0.43). Treatment with memantine at 0.3 or 5 mg/kg increased these basal t-MeHA levels by 20 ± 7 and +52 ± 7%, respectively (175 ± 8 and 217 ± 9 versus 148 ± 5 ng/g; p < 0.001) (Fig. 4B).

**Effects of Single or Repeated Administrations of Memantine on [¹²⁵I]Iodoproxyfan, [³H]Mepyramine, and [³H]MK-801 Binding Sites in Mouse Brain.** A single intraperitoneal administration of memantine did not significantly change [¹²⁵I]iodoproxyfan at H₃Rs in the hypothalamus. When [¹²⁵I]iodoproxyfan was used at ~100 µM [i.e., at its Kᵦ, value in the various mouse brain regions (Morisset et al., 2000b)], specific binding after repeated administration of saline represented 31 ± 2 fmol/mg of protein in the cerebral cortex (64 ± 1% of total binding), 50 ± 4 fmol/mg of protein in the striatum (78 ± 1% of total), 25 ± 2 fmol/mg of protein in the hypothalamus (64 ± 1% of total), and 20 ± 2 fmol/mg of protein in the hippocampus (60 ± 3% of total). After repeated administration of memantine, specific binding remained unchanged in the cerebral cortex, striatum, and hippocampus (not shown) but was increased by 92 ± 4% (48 ± 3 versus 25 ± 2 fmol/mg of protein) in the hypothalamus. When [¹²⁵I]iodoproxyfan was used at 180 µM, a saturating concentration (Morisset et al., 2000b), similar data were found, with no change of specific binding (which represented 30–50% of total) in the cerebral cortex, striatum, and hippocampus but a strong increase of specific binding in the hypothalamus (by 141 ± 24%, p < 0.001; Fig. 5 and Table 2).

In agreement with other biochemical studies, the density of H₁R binding sites in mouse brain appeared lower than that of H₂R binding sites. When [³H]mepyramine was used at a saturating concentration (5 nM), its specific binding at H₁Rs represented 17 ± 3 fmol/mg of protein in the cerebral cortex, 9 ± 1 fmol/mg of protein in the striatum, 10 ± 1 fmol/mg of protein in the hypothalamus, and 11 ± 1 fmol/mg of protein in the hippocampus. A single intraperitoneal administration of memantine did not significantly change [³H]mepyramine binding at H₁Rs in the various mouse brain regions (Table 2). After a 2-day washout, repeated administration of memantine (5 mg/kg) did not change [³H]mepyramine binding in any region (Table 2). The density of NMDA-receptor binding sites, determined by using [³H]MK-801 at a saturating concentration (20 nM), also remained unchanged in the four

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**Fig. 3.** t-MeHA levels in mouse brain after a single intraperitoneal administration of memantine. Mice were sacrificed 90 min after intraperitoneal administration of saline, the standard H₃-receptor inverse agonist CPX (3 mg/kg), the standard NMDA receptor antagonist MK-801 (0.3 mg/kg), or memantine at increasing doses. Data are expressed as percentage change of control values. Data are means ± S.E.M. of values from 20 to 24 animals. *, p < 0.05; ***, p < 0.001 versus saline (Sal) (one-way ANOVA, Student-Newman-Keuls post hoc test).

**Fig. 4.** Changes in t-MeHA levels induced in mouse brain after repeated administration of memantine. Mice received saline or memantine at 5 mg/kg i.p. twice daily for 5 days. After either a 2-day (left) or a 7-day (right) washout (w.o.), changes in brain t-MeHA levels were evaluated 90 min after intraperitoneal administration of saline or memantine (at 0.3 or 5 mg/kg). Data are expressed as percentage change of control values. Data are means ± S.E.M. of values from 13 to 14 (2-day w.o.) and 13 to 16 (7-day w.o.) animals. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus the corresponding saline; n.s., nonsignificant; §, p < 0.001 versus saline pretreatment and treatment (saline/saline) (Fisher least significant difference post hoc test).
TABLE 2

Short- and long-term effects of memantine on H1R, H3R, and NMDAR binding sites in mouse brain

<table>
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<tr>
<td><strong>% of control</strong></td>
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*p < 0.001.

**Discussion**

This study shows that memantine increases brain t-MeHA levels, an index of histamine neuron activity, by antagonism of NMDARs. We reported previously that other NMDAR channel blockers, MK-801 and phencyclidine, also enhanced histaminergic activity (Faucard et al., 2006). Moreover, the magnitude of the increase was similar to that found with memantine and slightly lower than that induced by H3R inverse agonists.

In agreement with previous studies (Kornhuber et al., 1989; Gilling et al., 2009), memantine inhibited [3H]MK-801 binding to rat hippocampal NMDARs with an IC50 of ~1 μM. As reported (Gilling et al., 2009), it inhibited the effect of NMDA on Ca2+ influx with a lower potency that was suggested to be due to the strong voltage dependence of memantine and to the fact that cells were free to depolarize, in contrast to binding assays, in which no membrane potential occurs, and to electrophysiological experiments, in which membranes were clamped at ~70 mV.

The micromolar potency of memantine in vitro at NMDARs is in the same range as its blood and brain concentrations after administration of therapeutic doses (typically 20 mg/day) (Kornhuber and Quack, 1995; Hesselink et al., 1999). Moreover, taking into account this modest potency, the ED50 of memantine on ex vivo [3H]MK-801 binding (in the mg/kg range) confirmed its easy brain penetration and was within the concentration range of therapeutic relevance (up to 5 mg/kg) after short-term administration to rats (Parsons et al., 2007). Therefore, our data further support a significant occupation of NMDARs by memantine in vivo at therapeutically relevant doses. Whereas it was almost fully displaced by administration of MK-801, it is worth noting that [3H]MK-801 binding was only partially inhibited by administration of memantine, which likely reflects its fast unblocking kinetics (Gilling et al., 2009) leading to its rapid ex vivo dissociation in binding assays. In agreement, the IC50 of memantine on ex vivo binding, calculated from its ED50 and molecular weight, was ~8 μM (i.e., 10-fold lower than its in vitro potency).

Confirming that it increased brain t-MeHA levels by antagonism of NMDARs, a single administration of memantine increased t-MeHA levels with an ED50 of 0.3 mg/kg, very similar to that found in ex vivo [3H]MK-801 binding. When pretreatment at 5 mg/kg was performed twice daily for 5 days to fit with the protocol generally followed by patients, this ED50 value remained unchanged.

After pretreatment with memantine, basal t-MeHA levels...
Histamine neuron activity), were enhanced by 50% 2 days later and had returned to initial values 7 days later. This long-lasting activation of histamine neurons suggests genetic regulations induced by blockade of NMDARs. The H3R playing a critical role in vivo in the modulation of histamine neurons, we first investigated putative changes of the H3R protein and mRNA at the corresponding time point (i.e., 2 days after pretreatment with memantine). Surprisingly, whereas it has no significant potency at H3Rs, memantine increased [125I]iodoproxyfan binding at half-saturating or saturating concentrations (i.e., H3R density) (Morisset et al., 2000b). Moreover, this increase was restricted to the hypothalamus, in which histaminergic perikarya are located. The location of these H3Rs on histamine neurons themselves (autoreceptors) or on afferent nerve endings (heteroreceptors) remains unknown, inasmuch as many neuronal populations coexpress NMDARs and H3Rs, including histamine neurons (Faucard et al., 2006). Our qPCR analysis confirmed that this up-regulation resulted from an enhanced transcription. H3R mRNAs were also increased in hypothalamus, suggesting an increase in autoreceptor density. However, H3R mRNAs expressed in other areas, such as the cerebral cortex, striatum, and hippocampus (Pillot et al., 2002), were increased as well. It is unclear why increases in mRNAs did not yield increases

Fig. 6. Quantitative analysis of H3R, BDNF, NR1, and nicotinic α7R mRNA expression in mouse brain after repeated administration of memantine. Mice received saline (controls) or memantine (5 mg/kg i.p.), twice daily for 5 days, and were sacrificed after a 2-day washout. mRNA expression levels of each target in various brain regions were determined by qPCR and normalized to β-actin mRNA expression levels. Results expressed as percentages of controls are means ± S.E.M. of values obtained from eight animals per group (two separate experiments were performed with triplicate determinations for each animal). **, p < 0.01; ***, p < 0.001 versus control.

Fig. 7. RT-PCR analysis of H3R mRNAs in mouse brain after repeated administration of memantine. Mice received saline or memantine (5 mg/kg i.p.), twice daily for 5 days and were sacrificed after a 2-day washout. Total RNAs from various brain regions were digested with RNase-free DNase I to avoid any amplification of residual genomic DNA and were subjected to reverse transcription and PCR amplification (35 cycles). Several H3R cDNA fragments with sizes corresponding to mouse H3(445)R, H3(413)R, and H3(397)R isoforms were amplified.
in proteins in the corresponding brain areas 2 days after pretreatment. However, these mRNAs encode not only for intrinsic somatodendritic H3Rs but also for H3Rs present on efferent projections (Pillot et al., 2002). The differences observed between hypothalamus and other regions may therefore suggest differences in regulations between auto- and heteroreceptors and/or somatodendritic H3Rs. Alternatively, these differences might have resulted from the existence of multiple functional and truncated H3R isoforms. However, the mRNA expression of the mouse H3.4,5,7,13, and H3.3,7,13,17 isoforms (Rouleau et al., 2004) was clearly increased not only in the hypothalamus but also in the cerebral cortex, hippocampus, and striatum, showing that all functional isoforms are similarly up-regulated and are also up-regulated in nonhistaminergic neurons. Because it probably deals with inhibitory autoreceptors, the increase in hypothalamic H3R density 2 days after treatment cannot mediate the histamine neuron hyperactivity. It is, rather, a compensatory response tending to reverse this hyperactivity, as shown by its disappearance 5 days later.

Although our data strongly suggest activation by memantine of a signaling cascade between NMDARs and the H3R gene, it is worth noting that administration of memantine to rats has been shown to up-regulate several genes that are both dependent and independent of NMDAR blockade (Marvanová et al., 2004). This important issue requires further analysis of the effect of repeated administrations of other NMDAR antagonists on histamine neuron activity and H3R mRNA expression. Among these genes, BDNF was previously shown to be up-regulated by memantine (Marvanová et al., 2001; Jantas et al., 2009; Lockrow et al., 2010). However, after pretreatment and 2-day washout, BDNF mRNAs were up-regulated only in the hippocampus. This BDNF up-regulation probably accounts for the NR1 subunit up-regulation, also restricted to hippocampus. BDNF up-regulates NMDARs in hippocampal neurons (Caldeira et al., 2007), and an up-regulation of NR1 subunits directly triggered by blockade of NMDARs would have been expected to occur in all of the brain areas. Although this hippocampal BDNF/NR1 up-regulation probably underlies, at least in part, the improvement of cognition and learning induced by memantine in patients with AD (Monteggia et al., 2004), its relationship with the increase in histaminergic neuron activity may have the potential to improve cognitive deficits of AD. In addition to its cholinergic and glutamatergic systems, the histaminergic system seems therefore to be an interesting target for the symptomatic treatment of the disease, as much as we showed that histaminergic neurons can still be activated in AD (Motawaj et al., 2010). To our knowledge, the present study is the first to report the effect of a repeated treatment by memantine on a monoaminergic system. In a microdialysis study, its short-term administration induced region specific changes in extracellular acetylcholine, dopamine, noradrenaline, and serotonin (Shearman et al., 2006). In addition to its cognitive properties, which are observed after as little as 2 weeks of treatment (Johnson and Kotermanski, 2006), it is generally accepted that memantine slows the long-term progression of AD through its neuroprotective effects (Parsons et al., 1999; Johnson and Kotermanski, 2006; Lipton, 2006). In addition to their cognitive effects, histamine neurons have also neuroprotective effects in the immature brain (Kukko-Lukjanov et al., 2006). However, whether activation of the histaminergic system may contribute to the neuroprotective effects of memantine remains to be explored.

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Participated in research design: Motawaj and Arrang.
Conducted experiments: Motawaj, Burban, and Davenas.
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