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 neuron activity. The H4 receptor (H4R), 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 data from numerous pharmaceutical companies suggest that they should improve cognitive deficits encountered in Alzheimer’s disease (AD). The Aβ plaques and neurofibrillary degeneration in AD suggest that the drug exerts its beneficial effects on cognitive deficits of Alzheimer’s disease, at least partly, by activating histamine neurons.

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ABBREVIATIONS: hH1R, human H1 receptor; hH2R, human H2 receptor; hH3R, human H3 receptor; hH4R, rat H4 receptor; t-MeHA, tele-methylhistamine; AD, Alzheimer’s disease; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; MK-801, dizocilpine maleate; HEK, human embryonic kidney; JNJ 7777120, 5-chloro-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole; BZQ, p-benzoquinone; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; ANOVA, analysis of variance; CPX, ciprofloxacin; BDNF, brain-derived neurotrophic factor; NR1, NMDA-receptor subunit 1; α7R, α7 receptor.
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; Lipton, 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; Lipton, 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 H1Rs, memantine at increasing concentrations was added in the presence of 3 to 5 nM [3H]histamine 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 H1R-mediated inhibition of cAMP accumulation, the effects of memantine were studied using HEK-293 cells expressing either the human [HEK(hH1R)] or the rat [HEK(rH1R)] 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 H1 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 50 μM [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) (Janvier, Le Genest-Saint-Ise, 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 106 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 Ca2+, cells were loaded for 45 min at 37°C with the calcium dye Fluor-4, acetoxyethyl ester (5 μM Fluo4-AM; Molecular Probes, Eugene, OR) and excited at 488 nm in the presence of 50 μM Fluo-4/AM. In this condition, Fluo-4/AM was converted into the nonfluorescent acid, which is then esterified by the esterase activity of the cells and converted back to the fluorescent form in the cytosol. The required drug solutions were then injected and the fluorescence (F) induced by intracellular calcium ([(Ca2+)]c) mobilization was measured over a 2-min period. The maximal fluorescence intensity (Fmax) was determined in a fluorometric plate reader by using a decrease in F during the drug application. The percentage of Fmax was determined by subtraction of the control (0%) from the normalized fluorescence (0%).

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medium with 2 nM \(^{3}H\)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 (Millipore, Billerica, MA). Specific binding was defined as that inhibited by 1 \(\mu M\) 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. Membranes from various mouse brain regions were prepared. After the last centrifugation, aliquots of the membrane suspension (~50 \(\mu g\) of protein) were incubated for 15 min at 25°C with 20 nM \(^{3}H\)MK-801 (final volume, 200 \(\mu l\)). Specific binding was again determined using MK-801 (1 \(\mu M\)).

**Ex Vivo \(^{3}H\)MK-801 Binding.** Mice were sacrificed 30 min after intraperitoneal injection of memantine at increasing doses. The forebrain was homogenized with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) in 40 volumes of 5 mM Tris-HCl buffer, pH 7.4 at 4°C. Aliquots of the membrane suspension (200 \(\mu g\) of protein) were incubated for 15 min at 25°C with 2 nM \(^{3}H\)MK-801. Incubations were stopped, and specific binding was defined as described above.

**Brain tele-Methylhistamine Levels.** Drugs dissolved in saline solution (0.9% NaCl (w/v)) were administered intraperitoneally to mice. For single treatments, mice were sacrificed 90 min after administration of drugs. For repeated treatments by memantine, mice received saline or memantine (5 mg/kg) twice daily for 5 days, and after a 2-day or a 7-day washout, they were sacrificed 90 min after administration of saline or memantine. After the last administration, animals were sacrificed by decapitation. The total brain was dissected out and homogenized in 10 volumes (w/v) of ice-cold perchloric acid (0.4 N). The clear supernatant was stored at −20°C immediately after centrifugation (4000g for 20 min). t-MeHA levels were determined using an enzyme immunoassay derived from a radioimmunoassay described previously (Garberg et al., 1992). In brief, t-MeHA of the sample was derivatized with \(^{12}C\)-benzoinoquinone (BZQ; 2.8 mg/ml). The reaction was allowed to proceed at pH 7.9 for 3 h; then, 2 M glycine was added to eliminate the excess of BZQ. The derivatized extract was mixed with t-MeHA-BZQ-Leu-Tyr-acytycholinesterase as a tracer and an antiserum raised in rabbits against t-MeHA conjugated with bovine serum albumin via \(^{12}C\)-benzoinoquinone in a plate (Immuno-Plate Maxi-Sorp Surface; Nunc, Roskilde, Denmark) pretreated with swine antirabbit IgG (Cayman Chemical, Ann Arbor, MI). After incubation for 16 h at 15°C, plates were washed and the substrate for acetylcholinesterase, Ellman’s reagent, was added. After 5 h, the optical density was measured with a microplate reader (MR 5000; Dynatech, Horsham, PA) at 405 nm. The limit of detection was 5 pg of t-MeHA.

**\(^{[12]H}\)Iodoproxyfan Binding.** The density of HI\(_{3}\)R binding sites in various mouse brain regions was investigated using \(^{[12]H}\)iodoproxyfan, a potent and selective HI\(_{3}\)R radioligand (Ligneau et al., 1994). Mice were sacrificed 90 min after administration of saline or memantine (5 mg/kg i.p.), or they received saline or memantine (5 mg/kg i.p.) twice daily for 5 days and were sacrificed after a 2-day washout. Tissues from various mouse brain regions were homogenized with a Polytron homogenizer in ice-cold buffer (50 mM Na\(_{2}\)HPO\(_{4}\)/KH\(_{2}\)PO\(_{4}\), pH 7.5). After centrifugation (12,000 g for 30 min at 4°C), the pellet was suspended in ice-cold binding buffer, and assays were performed as described previously (Ligneau et al., 1994). Aliquots of membrane suspension (~20 \(\mu g\) of protein) were incubated for 60 min at 25°C with 180 pM \(^{[12]H}\)iodoproxyfan (final volume, 200 \(\mu l\)). The specific binding was defined as that displaced in the presence of the selective HI\(_{3}\)R agonist imetit (1 \(\mu M\)). Incubations were stopped by rapid filtration under vacuum through Whatman GF/B filters. Radioactivity present on the filters was directly counted using a gamma counter (Clinigamma 1272; PerkinElmer Life and Analytical Sciences).

**\(^{[3}H\)Mepyramine Binding.** The selective HI\(_{3}\)R radioligand \(^{[3}H\)mepyramine was used to evaluate the density of HI\(_{3}\)R binding sites in mouse brain. Mice were sacrificed 90 min after administration of saline or memantine (5 mg/kg i.p.), or they received saline or memantine (5 mg/kg i.p.) twice daily for 5 days and were sacrificed after a 2-day washout. Tissues from various mouse brain regions were homogenized with a Polytron homogenizer in ice-cold buffer (50 mM Tris-HCl, pH 7.4). After centrifugation (12,000 g for 30 min at 4°C), the pellet was suspended in ice-cold binding buffer. Aliquots of membrane suspension (~40 \(\mu g\) of protein) were incubated for 30 min at 25°C with 3 to 5 nM \(^{[3}H\)mepyramine (final volume, 200 \(\mu l\)). The nonspecific binding was determined using triprolidine (2 \(\mu M\)). Incubations were stopped by rapid filtration under vacuum through Whatman GF/B filters pretreated with 0.3% polyethylenimine. Radioactivity present on the filters was determined by liquid scintillation spectrometry.

**RNA Isolation.** Mice received saline (controls) or memantine (5 mg/kg i.p.) twice daily for 5 days and were sacrificed after a 2-day washout. Tissues dissected 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). Real-Time PCR. cDNAs were synthesized from total RNAs by reverse transcription using an Omniscript RT kit (QIAGEN). In brief, 1 \(\mu l\) 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 \(\mu M\) anchored oligo-dt (Thermo Fisher Scientific, Epson, Surrey, UK), 10 units of Nasin Ribonuclease inhibitor (Promega, Madison, WI), and 4 units of Omniscript reverse transcriptase (QIAGEN) were then added. The 20- \(\mu l\) 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- \(\mu l\) reaction volume using 25 ng of cDNA, 1 \(\times\) TaqMan Universal PCR Master Mix without AmpErase uracil N-glycosylase (Applied Biosystems), and 1 \(\times\) 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 \(\beta\)-actin content and expressed as percentages of control sample. Relative quantification was calculated using the 2\(^{-\Delta\Delta Ct}\) method. For quantification of \(H_{3}\)R mRNA expression, the amplified 83-base pair fragment was located at boundaries of exons 2 and 3 of the mouse \(H_{3}\)R 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 \(H_{3}\)R forward, 5’-TGG TAC GGT TGG CTT GTG GT-3’; reverse, 5’-CAT CCT CAT GCG CTT CTC CA-3’; \(\beta\)-actin: forward, 5’-GTG TGA TGG TGG GAA TGG GTG GT-3’; reverse, 5’-ACG CAC GAT TTC 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 \(H_{3}\)R primers described above were based on the

### Table 1

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<th>Gene RefSeq Assay ID</th>
<th>Amplicon Length</th>
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<tr>
<td>(\beta)-Actin</td>
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<tr>
<td>(H_{3})R</td>
<td>NM_133848</td>
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<tr>
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<td>NRI</td>
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<td>Nicotinic (\alpha)R</td>
<td>NM_007390</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 H₄ 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, ED₅₀, and their S.E.M. The apparent affinity constant (Kₗ) 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): Kₗ = IC₅₀/1 + (S/Kᵩ), where S represents the concentration (2 nM) and Kᵩ 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. Schumack (Freie Universität, Berlin, Germany). MK-801, histamine, NMDA, tripropidil, imetit, and tele-methylhistamine were from Sigma-Aldrich (St Louis, MO). [¹²⁵I]Iodoproxyfan (2200 Ci/mmol), [³H]methyamine (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(h₂R) cells, specific [¹²⁵I]iodoproxyfan binding to membranes from HEK(h₄R) cells, or specific [³H]histamine binding to membranes from HEK(h₄R) cells (not shown).

In cAMP assays, histamine enhanced cAMP formation in HEK(h₄R) 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 H₄R, the reference compound thioperamide behaved as an inverse agonist and strongly enhanced (by ~600%) cAMP formation in HEK(rH₄R) and HEK(h₄R) 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 Kᵩ 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 H₄R 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

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**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).

**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.
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 ED$_{50}$ 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 tele-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 ($F_{5,75} = 25.46$; 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 ($F_{5,79} = 38.39$; 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 $[^{125}]$Iodoproxyfan, $[^{3}H]$Mepyramine, and $[^{3}H]$MK-801 Binding Sites in Mouse Brain.** A single intraperitoneal administration of memantine did not significantly change $[^{125}]$iodoproxyfan at H$_{3}$Rs in the hypothalamus. When $[^{125}]$iodoproxyfan was used at ~100 μM [i.e., at its $K_{D}$ 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 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 $[^{125}]$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 H$_{3}$R binding sites in mouse brain appeared lower than that of H$_{2}$R binding sites. When $[^{3}H]$mepyramine was used at a saturating concentration (5 nM), its specific binding at H$_{3}$Rs 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 $[^{3}H]$mepyramine binding at H$_{3}$Rs in the various mouse brain regions (Table 2). After a 2-day washout, repeated administration of memantine (5 mg/kg) did not change $[^{3}H]$mepyramine binding in any region (Table 2). The density of NMDA-receptor binding sites, determined by using $[^{3}H]$MK-801 at a saturating concentration (20 nM), also remained unchanged in the four
TABLE 2

<table>
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<th>Treatment</th>
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<th>Striatum</th>
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<th>Hippocampus</th>
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<td>H3R</td>
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<td>103 ± 13</td>
<td>115 ± 7</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>H3R</td>
<td>90 ± 10</td>
<td>88 ± 9</td>
<td>100 ± 4</td>
<td>101 ± 12</td>
</tr>
<tr>
<td>Repeated treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3R</td>
<td>241 ± 24</td>
<td>105 ± 14</td>
<td>105 ± 19</td>
<td>95 ± 17</td>
</tr>
<tr>
<td>H3R</td>
<td>109 ± 11</td>
<td>110 ± 14</td>
<td>99 ± 11</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>NMDAR</td>
<td>134 ± 32</td>
<td>117 ± 11</td>
<td>108 ± 4</td>
<td>117 ± 13</td>
</tr>
</tbody>
</table>

* p < 0.001.

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 [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 of mouse brain H3R isoforms was investigated by RT-PCR analysis 2 days after repeated administrations of memantine (5 mg/kg). The obtained length of the three amplified products (~700, 600, and 550 base pairs) corresponded to the mouse H3(445)R, H3(413)R, and H3(397)R isoforms reported previously (Rouleau et al., 2004). In agreement with the regional distribution of H3R transcripts and binding sites (Pillot et al., 2002; Rouleau et al., 2004), mRNA expression of H3R isoforms after saline administration was higher in the cerebral cortex and striatum than in hippocampus and hypothalamus (Fig. 7). The mRNA expression of the three H3R isoforms was clearly increased in the cerebral cortex, hippocampus, hypothalamus, and striatum after repeated administrations of memantine (Fig. 7).

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.

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After pretreatment with memantine, basal t-MeHA levels

Effects of Repeated Administrations of Memantine on mRNA Expression of H3R, BDNF, NR1, and Nicotinic α7R. Quantitative real-time PCR was used to quantify the expression of H3R, brain-derived neurotrophic factor (BDNF), NMDA-receptor subunit 1 (NR1) subunit and nicotinic α7 receptor (α7R) mRNAs in various mouse brain regions 2 days after repeated administrations of memantine (5 mg/kg). Compared with controls (100%), H3R mRNA expression was significantly increased in cerebral cortex, hippocampus, striatum, and hypothalamus, (1290 ± 150, 880 ± 120, 350 ± 50, and 310 ± 60%, respectively) (Fig. 6A). BDNF mRNA expression significantly increased only in hippocampus (370 ± 60%) but did not change in cerebral cortex, striatum, or hypothalamus (Fig. 6B). NR1 mRNA expression displayed a similar pattern and was significantly increased in hippocampus (1000 ± 200%) but not in the other three regions (Fig. 6C). Memantine did not significantly change nicotinic α7R mRNA expression in any mouse brain region (Fig. 6D).

Effects of Repeated Administrations of Memantine on mRNA Expression of H3R Isoforms. mRNA expression

Effects of Repeated Administrations of Memantine on mRNA Expression of H3RIsoforms. mRNA expression...
(i.e., 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 H3R(R), H3R(413)R, and H3R(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 H3R subunits but also for H3Rs present on effenter 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 H3R. Alternatively, these differences might reflect the result of the existence of multiple functional and truncated H3R isofoms. However, the mRNA expression of the mouse H3_	ext{A455R}, H3_	ext{A413R}, and H3_	ext{A307}R isoforms (Rouleau et al., 2004) was clearly increased not only in the 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 underlying, 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 histamine neuron activity and H3R mRNA expression remains to be explored. It is noteworthy that although histamine neurons selectively express α7-nicotinic receptors (Uteshev et al., 2002), and memantine has been shown to block α7-nicotinic receptors in vitro (Aracava et al., 2005), no up-regulation of these receptors was observed in any region, suggesting that α7-nicotinic receptors are not involved in the effect of memantine on histamine neuron activity.

Among the explanations for the paradox of cognitive benefits of memantine in AD, a readjustment of the balance between inhibition and excitation in the brain has been hypothesized (Schmitt, 2005). Our data support such a hypothesis, because histamine neurons do express NMDA receptors (Faucaur et al., 2006), but are activated, and not inhibited, by memantine. Therefore, the increase in histamine neuron activity resulting from blockade of NMDARs present on inhibitory neuronal inputs to the tuberomammillary nucleus. Among the latter, sleep-activated GABAergic neurons arising from the ventrolateral preoptic area play a major role in the regulation of histamine neurons (Haas and Panula, 2003). In agreement with an indirect effect, activation of NMDARs in the diagonal band of Broca, the lateral preoptic area, and the anterior hypothalamus evoked not only excitatory responses but also inhibitory responses of histaminergic neurons (Yang and Hatton, 1997). These changes in firing rates of tuberomammillary neurons were interpreted to result from glutamate activation of neurons with not only excitatory but also inhibitory (GABAergic) connections to histamine neurons.

The increase in brain t-MeHA levels observed after single or repeated administration of therapeutic doses of memantine strongly suggests that the drug exerts its beneficial effects on cognitive deficits of AD, at least in part, by activating histamine neurons. The drug tacrine, which is used in long-term palliative treatment of AD, enhances histaminergic neurotransmission by inhibiting histamine-N-methyltransferase, the main histamine-metabolizing enzyme in the brain, more potently than acetylcholinesterase (Morisset et al., 1996). H3R inverse agonists that enhance histamine release (Morisset et al., 2000a) raise considerable interest as potential agents to improve cognitive deficits of AD. In addition to the cholinergic and glutamatergic systems, the histaminergic system seems therefore to be an interesting target for the symptomatic treatment of the disease, inasmuch 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 microdiary 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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Authorship Contributions

Participated in research design: Motawaj and Arrang.
Conducted experiments: Motawaj, Burban, and Davenas.
Performed data analysis: Motawaj and Burban.
Wrote or contributed to the writing of the manuscript: Motawaj, Davenas, and Arrang.

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