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**HISTAMINE POTENTIATES N-METHYL-D-ASPARTATE
RECEPTORS BY INTERACTING WITH AN ALLOSTERIC SITE
DISTINCT FROM THE POLYAMINE-BINDING SITE.**

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JPET # 158543

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List of nonstandard abbreviations CGP39653: D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid; EPSCs: excitatory postsynaptic currents; GBR12909: (1-[2- [bis(4-fluorophenyl)-methoxy]ethyl]-4-(3-phenylpropyl)piperazine; HA: histamine; NMDA_(HA)R: histamine site of the NMDAR; 7-KYNA: 7-kynurenate; MK-801: dizocilpine ((+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine); NMDARs: N-methyl-D-aspartate receptors; PEA: 2-pyridylethylamine; PEAQX: [[[1S)-1-(4-Bromophenyl)ethyl]amino] (1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxaliny]methyl] phosphonic acid tetrasodium hydrate; *tele*-MeHA: *tele*-methylhistamine;

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ABSTRACT

Histamine potentiates activation of native and recombinant *N*-methyl-D-aspartate receptors (NMDARs), but its mechanisms of action and physiological functions in the brain remain controversial. Using four different models, we have further investigated the histamine-induced potentiation of various NMDAR-mediated responses. In single cultured hippocampal neurons, histamine potentiated NMDA currents. It also potentiated the NMDA-induced increase in intracellular calcium in the absence, as well as with saturating concentrations, of exogenous D-serine, indicating both glycine-dependent and glycine-independent components of its effect. In rat hippocampal synaptosomes, histamine strongly potentiated NMDA-induced [³H]noradrenaline release. The profile of this response contained several signatures of the histamine-mediated effect at neuronal or recombinant NMDARs. It was NR2B selective, being sensitive to micromolar concentrations of ifenprodil. It was reproduced by *tele*-methylhistamine, the metabolite of histamine in brain, and it was antagonised by impromidine, an antagonist/inverse agonist of histamine on NMDA currents. Up to now, histamine was generally considered to interact with the polyamine site of the NMDAR. However, spermine did not enhance NMDA-induced [³H]noradrenaline release from synaptosomes, and the potentiation of the same response by *tele*-methylhistamine was not antagonized by the polyamine antagonist arcaine. In hippocampal membranes, like spermine, *tele*-methylhistamine enhanced [³H]CGP39653 binding to the glutamate site. In contrast, spermine increased non-equilibrium [³H]MK-801 binding, and suppressed [³H]ifenprodil binding, whereas histamine and *tele*-methylhistamine had no effect. In conclusion, the histamine-induced potentiation of NMDARs occurs in the brain under normal conditions. Histamine does not bind to the polyamine site, but to a distinct entity, the so-called histamine site (NMDA_(HA)R) of the NMDAR.

JPET # 158543

INTRODUCTION

Histamine neurons constitute a long and highly divergent system arising from the tuberomammillary nucleus in the posterior hypothalamus and projecting in a diffuse manner to many cerebral areas. The function of these neurons in the modulation of physiological processes such as arousal or cognitive functions is well documented, but their involvement in brain disorders remains poorly understood. In the brain, the effects of histamine are mediated by three histamine receptor subtypes (H_1 , H_2 and H_3), which are all G protein coupled receptors. Brain histamine is metabolized via transmethylation into *tele*-methylhistamine (*tele*-MeHA) catalyzed by histamine *N*-methyltransferase, and this metabolite is devoid of any activity at histamine receptors (Brown et al., 2001).

More than fifteen years ago, histamine has been reported to act as a positive allosteric modulator of the *N*-methyl-D-aspartate receptor (NMDAR). Histamine potentiated NMDA currents in isolated (Vorobjev et al., 1993), and cultured (Bekkers, 1993) hippocampal neurons. Its effect resulted from a direct interaction with the NMDAR, the potentiation being observed on recombinant NMDARs. Moreover, it was selective for receptors containing NR1 variants lacking exon 5 with NR2B subunits (Williams, 1994b; Williams, 1995).

However, in spite of the potential therapeutic interest of allosteric modulators of the NMDAR for the treatment of various neurological or psychiatric disorders (Paoletti and Neyton, 2007), very few studies have been performed on this histamine-induced potentiation. One probable explanation for this rather limited interest is that its physiological relevance has remained unclear. Indeed, at the physiological pH of 7.4, histamine failed to potentiate the NMDA component of EPSCs in rat and mouse hippocampal slices (Saybasili et al., 1995; Yanovsky et al., 1995; Bekkers et al., 1996). Although poorly characterized, some other responses could however reflect an interaction of histamine with NMDARs. Among them, histamine facilitated the NMDA-induced depolarization of projection neurons in cortical

JPET # 158543

slices (Payne and Neuman, 1997). Phase shifts of the circadian clock induced by histamine in slices of the hamster suprachiasmatic nucleus were NMDA dependent (Meyer et al., 1998). Histamine might also act through NMDARs to facilitate the induction of long-term potentiation (Brown et al., 1995), and to cause long-lasting increases of excitability (Selbach et al., 1997) in the CA1 region of rat hippocampal slices. *In vivo*, histamine reversed spatial memory deficits induced in rats by the NMDAR glycine site antagonist 7-chlorokynurenic acid (Nishiga and Kamei, 2003), and facilitated a NMDA-dependent long-term potentiation in the visual cortex (Kuo and Dringenberg, 2008).

Another explanation for the limited interest in the histamine-induced potentiation likely results from the fact that histamine was generally thought, not to interact with a selective site, but with the polyamine allosteric site of the NMDAR (Brown et al., 2001). Histamine- and spermine-induced potentiations shared many similarities, including NR2B selectivity (Williams, 1994b; Williams et al., 1994; Williams, 1995), pH-dependence (Saybasili et al., 1995; Traynelis et al., 1995; Yanovsky et al., 1995), and voltage-independence (Vorobjev et al., 1993; Williams, 1994b; Williams, 1994a). In other studies, the effect of histamine was occluded by spermine (Vorobjev et al., 1993), or inhibited by the polyamine antagonist arcaine (Kuo and Dringenberg, 2008; Watanabe et al., 2008). However, in all these models, histamine and spermine were both active, and whether they bound to a common site, or to separate sites with allosteric interactions, remained unclear.

In this study, we further investigated and characterized the histamine-mediated potentiation of native NMDARs by using four different models: NMDAR-mediated currents and intracellular calcium in single cultured neurons, NMDA-mediated [³H]noradrenaline release from synaptosomes, and three NMDAR binding assays ([³H]MK-801, [³H]CGP39653 and [³H]ifenprodil binding) to extensively washed hippocampal membranes.

METHODS

Primary neuronal cultures.

The hippocampus was removed from 18-day-old embryos of male Wistar rats (Janvier, Le Genest-St-Isle, France). Cells were dissociated with a fire-narrowed Pasteur pipette and were seeded on 12-well culture dishes (100, 000 cells/well containing 1 ml of medium), previously coated with poly D-lysine. When intracellular Ca^{2+} was measured, cells were plated on glass slides previously coated with poly L-ornithine (15 $\mu\text{g/ml}$) and laminin (2 $\mu\text{g/ml}$) at a seeding density of 1 million cells/ml.

After removal of the coating solution, cells were seeded in neurobasal medium supplemented with B27 (1:50), GlutaMAX-I- (2 mM) and penicillin–streptomycin (5 IU/ml and 5 $\mu\text{g/ml}$, respectively). In these conditions, cultures of neurons were favoured at the expense of glial cells. Neurons were maintained for 2 to 4 weeks at 37°C in a humidified atmosphere containing 5% CO_2 . For intracellular Ca^{2+} measurement, neurons were maintained for 7 to 11 days *in vitro* (DIV) without medium change.

Whole-cell patch clamp.

Ionic currents were recorded within large pyramidal neurons using the whole-cell patch clamp technique at room temperature (21-22°C). Pipette electrodes were made from 1.5 mm (outer diameter) borosilicate glass tubes with a resistance of 2 to 3 $\text{M}\Omega$ when filled with the internal solution. The membrane potential was clamped at -50 mV. The whole-cell current recording started 5 to 10 min after membrane rupture in order that the cell interior adequately equilibrates with the pipette solution. Currents through the electrode were recorded with an Axopatch-1D amplifier (Axon Instruments, Union City, CA), filtered at 2 kHz, and sampled at 10 kHz in a PC-based data acquisition system that also provided preliminary data analysis.

JPET # 158543

A rapid perfusion system was monitored for drug application. The external solution for recording whole-cell currents contained 140 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 12 mM HEPES acid, 12 mM HEPES sodium, and 33 mM D-glucose. Tetrodotoxin (5 μM) was added to eliminate the voltage-gated sodium channel currents. Glycine 5 μM was added to the external solution. The pH was 7.3 and the osmolarity was adjusted to 300 mOsm with D-glucose. The internal pipette solution contained 100 mM CsF, 40 mM CsCl, 1 mM CaCl₂, 20 mM HEPES acid and 10 mM EGTA. The pH was adjusted to 7.3 with CsOH.

[³H]Noradrenaline release from rat hippocampal synaptosomes.

A crude synaptosomal fraction was prepared as previously described with minor modifications (Gray and Whittaker, 1962). Adult male Wistar rats (200-250g) were killed by decapitation. The hippocampus was rapidly removed and homogenized (Potter-Elvehjem glass, 8 up-down strokes) in 40 volumes of 0.32 M sucrose. The homogenate was first centrifuged (10 min, 1000 × g) to remove nuclei and cellular debris. Synaptosomes were isolated from the supernatant by a second centrifugation (12,000 ×g for 20 min). The synaptosomal pellet was then suspended in modified Krebs-Ringer bicarbonate medium of the following composition (mM): 120 NaCl, 0.8 KCl, 1.2 KH₂PO₄, 1.3 CaCl₂, 1.2 MgSO₄, 27.5 NaHCO₃, 10 glucose; 0.06 ascorbic acid, 0.03 EDTA; pH 7.4 and gassed with O₂/CO₂ (95/5).

The synaptosomal preparation maintained under a constant stream of O₂/CO₂ (95/5), was incubated for 1 hour at 37°C with [³H]noradrenaline (final concentration 30 nM). Synaptosomes were then extensively washed by four successive suspensions in fresh oxygenated medium in which Mg²⁺ was omitted, and centrifugations. After the last centrifugation, synaptosomes were suspended in the same Mg²⁺-free medium at a concentration of 0.7 mg protein/mL

JPET # 158543

Aliquots of the synaptosomal suspension (200 µg of protein) were distributed in microtubes kept at 37°C and incubated in a final volume of 500 µl with 200 µM NMDA, 1 µM glycine, and, when required, drugs to be tested. The standard H₃ receptor inverse agonist thioperamide was also added at a saturating concentration (1 µM) in all incubations in order to avoid the involvement of H₃ heteroreceptors modulating [³H]noradrenaline release (Schlicker et al., 1989). After 3 min, incubations were stopped by centrifugation, and the amount of [³H]noradrenaline released in the medium was determined by liquid scintillation spectrometry.

[³H]MK-801, [³H]CGP39653 and [³H]ifenprodil binding to rat hippocampal membranes.

Three different NMDAR binding assays were used. The open-channel blocker [³H]MK-801 was used to label its intra-channel site. The high-affinity and competitive antagonist [³H]CGP39653 was used to label the NMDA recognition site. The non-competitive NR2B antagonist [³H]ifenprodil was used to label the ifenprodil site of NR2B-containing NMDARs.

For [³H]MK-801 binding, hippocampal membranes from adult Wistar rats were prepared and extensively washed by four successive centrifugations in 5mM Tris-HCl buffer (pH 7.4). After the last centrifugation, aliquots of the membrane suspension (~200 µg protein) were incubated for 15 min at 25°C in 0.5 ml of the same medium with 2nM [³H]MK-801 and, when required, the drugs to be tested. In association studies, incubations varied from 1 to 60 min. Incubations were stopped by addition of ice-cold buffer, and rapid filtration over vacuum onto Millipore GF/B filters. Specific binding was defined as that inhibited by 1 µM MK-801. Radioactivity present on the filters was determined by liquid scintillation spectrometry.

For [³H]CGP39653 binding, hippocampal membranes were prepared and washed as described for [³H]MK-801 binding. The membranes (150-250 µg protein) were incubated for

JPET # 158543

60 min at 4°C (1 ml final volume) with 3nM [³H]CGP39653 and, when required, the drugs to be tested. Incubations were stopped by addition of ice-cold buffer, and rapid filtration over vacuum onto Millipore GF/B filters. Specific binding was defined as that inhibited by 100 μM glutamate and represented 64 ± 3 fmol/mg protein.

For [³H]ifenprodil binding, extensively washed membranes were suspended in 50mM Tris-HCl buffer (pH 7.4). The membranes (250-350 μg protein) were incubated for 60 min at 4°C (1 ml final volume) with 2nM [³H]ifenprodil and, when required, the drugs to be tested. All incubations were performed in the presence of 3 μM GBR12909 in order to saturate sigma sites. Incubations were stopped by addition of ice-cold buffer, and rapid filtration over vacuum onto Millipore GF/B filters. Specific binding was defined as that inhibited by 10 μM ifenprodil and represented 150 ± 20 fmol/mg protein.

Intracellular Ca²⁺ in single cultured neurons.

Cytosolic-free Ca²⁺ was monitored in pyramidal neurons cultured on glass slides by ratio imaging of the fluorescent Ca²⁺ probe Fura-2FAM. Cells were loaded for 30 min with 5 μM Fura-2F AM. After loading, the glass slide was placed in a perfusion chamber where cells were exposed to buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, glucose 1g/l). When required, neurons were exposed to tested compounds in the same buffer in which Mg²⁺ was omitted (Mg²⁺-free buffer) using a multichannel superfusion device. Neurons were alternatively excited at 340–380 nm using a Hamamatsu monochromator. Emission intensities were monitored at 510 nm. The camera dark noise was subtracted from the recorded crude image (camera and digitizing system were from Hamamatsu, Hamamatsu, Japan). Intracellular calcium concentrations corresponded to the ratio between fluorescence measured in single cells at 340 and 380 nm excitation wavelength (F₃₄₀/F₃₈₀), and results were expressed as per cent of the maximal ratio elicited by 20 μM NMDA and 10μM D-serine.

JPET # 158543

A glycine concentration as low as 10 nM being able to activate NMDA currents and being found in purified water and reagents (Ascher, 1990; Pittaluga and Raiteri, 1990), we used such a perfusion system in order to remove the endogenous glycine and D-serine present in the medium.

Analysis of Data.

The total curves were analyzed with an iterative least-squares method derived from that of Parker and Waud (Parker and Waud, 1971). Computer analysis was performed by nonlinear regression using a one-site cooperative model. The method provided estimates for IC₅₀ and EC₅₀ values and their SEM. Statistical analysis of the data was performed by one way ANOVA followed by a Student Newman Keuls *post hoc* test.

Radiochemicals and drugs.

[³H]Noradrenaline (48 Ci/mmol) was purchased from GE Healthcare, (Buckinghamshire, UK). [³H]MK-801 (22 Ci/mmol), [³H]CGP39653 (46 Ci/mmol) and [³H]ifenprodil (40 Ci/mmol) were from Perkin Elmer. NMDA, glycine, thioperamide, L-histidine, DL-2-Amino-5-phosphonopentanoic acid (AP5), ifenprodil, MK-801, RO 25-6981, PEAQX, spermine, arcaine, histamine, *tele*-methylhistamine were obtained from Sigma Aldrich (St Louis, MO USA). D-Serine, 7-chlorokynurenic acid and *pros*-methylhistamine were obtained from Tocris (Bristol, UK). Impromidine, burimamide and 4-methylimidazole were provided by SKF laboratories. Imetit, *N*^α-methylhistamine, *N*^α, *N*^α-dimethylhistamine were provided by C.R. Ganellin (University College, London, UK). (*R*)*α*-methylhistamine and 2-pyridinylethylimidazole were provided by W. Schunack (Freie Universität Berlin, Germany).

RESULTS

Potentiation of NMDA currents by histamine and *tele*-methylhistamine in cultured hippocampal neurons.

Histamine (HA, 50 μ M) added alone had no effect (data not shown), but increased NMDA currents generated by L-aspartate or NMDA (50 μ M) in hippocampal neurons (Figure 1A). Its effect considerably varied between neurons and 6 out of 21 (about 30 % of the total) did not respond to histamine. After analysis of the remaining 15 responsive neurons on three different parameters, histamine induced a mean potentiation of 20 ± 12 % of the relative current, of 22 ± 9 % of the peak of the current, and of 15 ± 13 % of the following plateau. The potentiation of the relative current varied from 9% up to 47% for the highest responses such as that shown in Figure 1A.

tele-Methylhistamine (*tele*-MeHA, 50 μ M) added alone had no effect but potentiated also NMDA currents with an effect very similar to that of histamine. It increased by 26 ± 13 % the relative current (values ranging from 8% to 44%), by 25 ± 10 % the peak of the current, and by 17 ± 15 % the plateau ($n = 7$ cells) (Figure 1A). Again, three neurons did not respond to *tele*-methylhistamine.

Histamine and *tele*-methylhistamine increased in a concentration-dependent manner the relative current with EC_{50} values of 8.8 ± 2.1 μ M and 7.2 ± 1.5 μ M, respectively (Figure 1B).

Characterisation of [3 H]noradrenaline release induced by NMDA from rat hippocampal synaptosomes.

When synaptosomes loaded with [3 H]noradrenaline, were incubated with increasing concentrations of NMDA in the presence of 1 μ M glycine in a Mg^{2+} -free medium, a concentration-dependent increase in tritium release was observed (Figure 2A). The effect of

JPET # 158543

NMDA was concentration-dependent with an EC₅₀ value of $8.2 \pm 2.8 \mu\text{M}$ and a maximal effect of $7,800 \pm 1,100$ dpm/mg of protein.

[³H]Noradrenaline release induced by 200 μM NMDA was reduced by ~80% when a physiological concentration of Mg²⁺ (1.2 mM) was added in the medium (Figure 2B). The effect of NMDA was also significantly antagonised (by ~70-100 %) by the selective open-channel blocker MK-801 (1 μM), by the competitive glutamate antagonist AP5 (20 μM), and by the glycine site antagonist 7-kynureate (7-KYNA, 100 μM) ($F_{(4,10)} = 14.70$; $p < 0.001$ in one way ANOVA) (Figure 2B). None of the antagonists had any effect on the basal (spontaneous) efflux of [³H]noradrenaline release (data not shown).

Potentialiation of NMDA-induced [³H]noradrenaline release by various histaminergic compounds.

The releasing effect of NMDA (200 μM) in hippocampal synaptosomes was differentially modulated by various histaminergic derivatives used (Figure 3A and Table 1). When tested at 100 μM , histamine and *tele*-methylhistamine increased by fourfold the effect of NMDA ($F_{(5,12)} = 21.41$, $p < 0.001$). *pros*-Methylhistamine (*pros*-MeHA or 3-MeHA), the histamine precursor, L-histidine, *N* ^{α} -methylhistamine and *N* ^{α} ,*N* ^{α} -dimethylhistamine had no effect. The standard H₃-receptor agonist (*R*) α -methylhistamine (*(R)* α -MeHA) added at 100 μM tended to enhance [³H]noradrenaline release, its effect becoming significant at higher concentrations (Figure 3A and Table 1).

Histamine and *tele*-methylhistamine enhanced NMDA-induced [³H]noradrenaline release in a concentration dependent manner with EC₅₀ values of $99 \pm 17 \mu\text{M}$ and $97 \pm 22 \mu\text{M}$, respectively. Their effect was calcium dependent, being totally abolished when calcium was omitted in the medium (not shown). Their maximal effect ($70,700 \pm 17,900$ and $57,000 \pm 6,100$ dpm/mg protein, respectively) indicated a six- to sevenfold maximal increase of the

JPET # 158543

effect of NMDA (Figure 3B). MK-801 and the selective NR2B antagonist ifenprodil inhibited in a concentration-dependent manner the effect of *tele*-methylhistamine (100 μ M) with a maximal inhibition of ~80% and IC₅₀ values of $0.27 \pm 0.17 \mu$ M and $0.14 \pm 0.04 \mu$ M, respectively (Figure 3C). The preferential NR2A antagonist PEAQX (1 μ M) (Auberson et al., 2002) had no significant effect (-8 +/- 11 %). The increase induced by *tele*-methylhistamine was partially decreased by the H₃-receptor agonist imetit at 100 μ M. It was also fully antagonised by impromidine, an antagonist of histamine on NMDA currents (Bekkers, 1993), with an IC₅₀ value of $15 \pm 3 \mu$ M (Figure 3C and Table 1). In contrast, it was not modified by 100 μ M bicuculline, a GABA_A receptor competitive antagonist (Figure 3C).

The preferential H₁-receptor agonist 2-pyridylethylamine (PEA) (EC₅₀ of $5.0 \pm 0.5 \mu$ M) and 4-methylimidazole (EC₅₀ of $50 \pm 28 \mu$ M) induced a maximal increase of release and therefore behaved as full agonists. (*R*) α -methylhistamine (EC₅₀ of $41 \pm 24 \mu$ M) and the H₂/H₃-receptor antagonist burimamide (EC₅₀ of $6 \pm 2 \mu$ M) behaved as apparent partial agonists, compared to histamine and *tele*-methylhistamine (Figure 4A and Table 1). The increase induced by PEA (100 μ M) was inhibited in a concentration-dependent manner by ifenprodil and impromidine (Figure 4B). Impromidine alone also decreased NMDA-induced release (data not shown).

Histamine and *tele*-methylhistamine also enhanced [³H]noradrenaline release from synaptosomes in the absence of NMDA. The effect of *tele*-methylhistamine at 100 μ M ($48,800 \pm 2,520$ dpm /mg protein) was antagonised (by 70-100 %, $p < 0.001$) by 1 μ M MK-801, 3 μ M ifenprodil and 60 μ M impromidine (Figure 5). However, *tele*-methylhistamine (300 μ M) failed to increase [³H]noradrenaline release induced by 20mM K⁺ (data not shown).

JPET # 158543

Compared effects of histamine and spermine on NMDA-induced [³H]noradrenaline release.

In contrast to *tele*-methylhistamine, spermine (10 μ M or 1mM), an endogenous ligand of the polyamine site, failed to significantly modify [³H]noradrenaline release induced by NMDA at 200 μ M (Figure 6). Furthermore, spermine did not modify the *tele*-methylhistamine effect, and the polyamine site antagonist arcaine did not significantly decrease the effect of *tele*-methylhistamine (Figure 6).

Compared effects of histamine and spermine on NMDAR binding assays in rat hippocampus.

The effects of spermine and histamine were first compared on the non-equilibrium [³H]MK-801 binding to extensively washed membranes, a validated model of NMDAR activation.

After a 15 min incubation, the total specific binding of [³H]MK-801 (2nM) to rat hippocampal membranes represented approximately 500 fmoles/mg protein (Figure 7A). Spermine (10 μ M) enhanced the binding by $391 \pm 10 \%$, $p < 0.001$. In contrast to spermine, histamine and *tele*-methylhistamine (100 μ M) did not significantly change [³H]MK-801 binding. Moreover, at the concentration of 1 mM corresponding to their maximal effect on [³H]noradrenaline release, histamine and *tele*-methylhistamine significantly decreased the binding (by $38 \pm 7 \%$, $p < 0.05$ and $46 \pm 3 \%$, $p < 0.01$, respectively) (Figure 7A). Although *tele*-methylhistamine and histamine at 100 μ M had no effect by themselves, they partially occluded the [³H]MK-801 binding stimulated by spermine (Figure 7B). The increase in binding induced by 5 μ M spermine was significantly decreased ($p < 0.001$) from $391 \pm 10 \%$ to $284 \pm 6 \%$ by histamine, and to $319 \pm 5 \%$ by *tele*-methylhistamine.

JPET # 158543

Ifenprodil inhibited [³H]MK-801 binding in a concentration-dependent manner. Analysis of its inhibition curve by nonlinear regression led to a mean IC₅₀ value of 8.9 ± 2.5 μM with a pseudo-Hill coefficient of 0.7 ± 0.1. This inhibition by ifenprodil remained unchanged in the presence of 100 μM histamine (n_H = 0.7 ± 0.1; mean IC₅₀ = 9.2 ± 2.5 μM (Figure 7C).

In kinetic studies, the slow kinetics of association of [³H]MK-801 (2 nM) was dramatically enhanced by spermine (10 μM) but was not modified by 50 μM histamine (Figure 8). Following a 1h incubation, glutamate enhanced binding of [³H]MK-801 (2 nM) with a potency that remained unchanged with *tele*-methylhistamine (50 μM) (EC₅₀s of 80 ± 21 nM and 63 ± 14 nM, respectively).

The effects of spermine and histamine were also compared on [³H]CGP39653 binding, a radioligand of the glutamate site. Glycine (30 μM), spermine (10 μM) and *tele*-methylhistamine (50 μM) significantly changed the binding of 3 nM [³H]CGP39653 ($F_{(2,36)} = 11.77, p < 0.001$). *Post-hoc* analysis indicated that [³H]CGP39653 binding was decreased by glycine (-47 ± 14%, $p < 0.01$), and increased by spermine (+44 ± 4%, $p < 0.001$) and *tele*-methylhistamine (+19 ± 3%, $p < 0.01$) (Figure 9A). In the presence of 30 μM glycine, spermine still induced a significant increase (+126 ± 13%, $p < 0.01$), whereas the increase induced by *tele*-methylhistamine (+28 ± 10%) became non significant (Figure 9B).

Spermine (10 μM) completely inhibited [³H]ifenprodil binding to hippocampal membranes, whereas histamine (100 μM and 1 mM) had no effect (Figure 10).

Effect of histamine on NMDA-induced increase in intracellular Ca²⁺ concentrations in single hippocampal neurons.

Variations of intracellular Ca²⁺ were measured in 23 single hippocampal neurons loaded with the fluorescent probe Fura-2F AM, and superfused with the drug-containing

JPET # 158543

buffer (Figure 11). NMDA (20 μ M) plus D-serine (10 μ M) markedly increased intracellular Ca²⁺ in all the neurons tested. This effect was further enhanced (by 20 \pm 2 %) by histamine (10-100 μ M) ($F_{(2,24)} = 26.56, p < 0.001$) (Figure 11A).

Neither NMDA (20 μ M), nor histamine (10 μ M), perfused alone without D-Serine elicited enhancement of intracellular Ca²⁺ concentrations (Figure 5B). However, both compounds added together significantly increased by 32 \pm 2 % intracellular Ca²⁺ concentrations ($F_{(3,38)} = 49.95, p < 0.001$) (Figure 11B).

DISCUSSION

This study shows that histamine potentiates NR2B-containing NMDARs in the brain by interacting with a functional site distinct from the polyamine site. The potentiation by histamine was previously observed in neurons, but not in complex models such as slices (Bekkers, 1993; Bekkers et al., 1996). Whatever the mechanisms underlying these differences, histamine could potentiate NMDA not only in neurons, but also in synaptosomes, a preparation enriched in nerve endings.

Supporting the presence of presynaptic NMDARs upon noradrenergic terminals (Pittaluga and Raiteri, 1992), NMDA induced [³H]noradrenaline release from synaptosomes in a Mg²⁺-free medium. This effect was sensitive to NMDAR blockers, and was enhanced by histamine. In most release experiments, as already observed (Pittaluga and Raiteri, 1990; Pittaluga and Raiteri, 1992), MK-801 and Mg²⁺ did not completely suppress the effect of NMDA. This is likely due to their slow association kinetics, together with the short incubation used in our assay.

The histamine-induced enhancement of NMDA currents remained poorly characterized because most compounds had been tested at one concentration only (Bekkers, 1993; Vorobjev et al., 1993; Williams, 1994b). However, two striking features were observed, i.e. the enhancement reproduced by *tele*-methylhistamine, the catabolite of histamine in the brain, and the antagonism of histamine by the H₂ agonist/H₃ antagonist impromidine (Bekkers, 1993; Williams, 1994b). We show that histamine and *tele*-methylhistamine are equipotent on NMDA currents. Their effects are selective of NMDARs, being found with L-aspartate or NMDA, but not with quisqualate or kainate (Vorobjev et al., 1993).

Compared to patch-clamp, the release model was more suitable for a proper characterization of the response because concentration-response curves could be more easily

JPET # 158543

obtained. The histamine-mediated increase in [³H]noradrenaline release displayed the same profile as that reported on NMDA currents. Firstly, *tele*-methylhistamine, which is not active at histaminergic receptors, potentiated NMDA-induced currents and [³H]noradrenaline release with the same potencies as histamine (102% and 122% respectively, Table 1). Secondly, in agreement with a NR2B selectivity (Williams, 1994b; Williams, 1995), the release of [³H]noradrenaline evoked by *tele*-methylhistamine was antagonized by the NR2B antagonist ifenprodil (Williams, 1993; Avenet et al., 1997), but not by PEAQX used at a concentration (1 μM) known to block only NR2A-containing receptors (Auberson et al., 2002). Thirdly, impromidine antagonized *tele*-methylhistamine (Table 1), and reduced NMDA-induced currents (Bekkers, 1993) and [³H]noradrenaline release, indicating that it acted in fact as an inverse agonist. Lastly, both currents (Bekkers, 1993; Williams, 1994b) and release were enhanced by the H₃ agonist (*R*)-α-methylhistamine, partially decreased by the H₃ agonist imetit, and unchanged by the histamine precursor, L-histidine, *N*^α-methylhistamine, and *pro*-methylhistamine (3-methylhistamine in Williams, 1994b) (Table 1).

GABA_A receptors, with which histamine and *tele*-methylhistamine interact (Saras et al., 2008), did not affect [³H]noradrenaline release from synaptosomes. GABA currents in hippocampal neurons are unaffected by histamine (Bekkers, 1993), and the effect of *tele*-methylhistamine on [³H]noradrenaline release was not antagonized by bicuculline. An interaction of histamine with monoamine transporters might lead to an enhanced [³H]noradrenaline release. However, the effect of histamine and *tele*-methylhistamine was suppressed in a calcium-free medium, indicating that their effect was different from an amphetamine-like effect. Moreover, no high-affinity histamine transporter exists in neurons, and lesions of noradrenergic neurons did not change [³H]histamine uptake in hippocampal slices (Smits et al., 1988). Also, histamine did not inhibit [³H]noradrenaline uptake in synaptosomes at concentrations up to 1 mM (Tuomisto and Tuomisto, 1980). Lastly, *tele*-

JPET # 158543

methylhistamine did not increase [³H]noradrenaline release induced in synaptosomes by 20mM K⁺. Therefore, the enhancement of release induced by histamine results from its interaction with the NMDAR, and not with other partners (Table 1).

In patch-clamp studies, the EC₅₀ of histamine (~ 10 μM) as an enhancer of NMDA currents was similar to that reported (Vorobjev et al., 1993; Williams, 1994b). However, histamine was tenfold less potent in synaptosomes (EC₅₀ = 99 ± 17μM, Table 1), which may result from differences between presynaptic adult vs postsynaptic embryonic NMDARs. Moreover, as shown (Vorobjev et al., 1993; Zwart et al., 1996; Meyer et al., 1998), some neurons did not respond to histamine, and their presence in synaptosomes might underestimate the potency of histamine. The magnitude of the histamine-induced potentiation of NMDARs considerably varied among systems. Histamine potentiated NMDA by 20% in single neurons, but increased by up to sevenfold NMDA-induced [³H]noradrenaline release from synaptosomes. This difference may be due to synaptic vs non synaptic preparations, because a large increase (by up to tenfold) was also observed on NMDA-mediated synaptic transmission (Bekkers, 1993).

Histamine and *tele*-methylhistamine alone had no effect on intracellular Ca²⁺ or NMDA currents. However, they still enhanced [³H]noradrenaline release in the absence of NMDA. Their effect was again suppressed by MK-801, ifenprodil and impromidine, confirming an interaction with NMDARs. Activation of NMDARs by endogenous glutamate was excluded, because NMDAR antagonists did not decrease basal release. Moreover, histamine had no effect with low concentrations of glutamate (Vorobjev et al., 1993; Williams, 1994b). More likely, synaptosomal NMDARs in Mg²⁺-free medium were already activated. Activation of NMDARs on noradrenergic terminals occurs by simply removing Mg²⁺ from the medium (Paudice et al., 1998). Moreover, the preparation of synaptosomes

JPET # 158543

itself leads to their partial depolarization (Saller and Salama, 1984), and activity of NR2B-containing NMDARs is enhanced by depolarization (Clarke and Johnson, 2008).

Up to now, histamine was considered to interact with the polyamine site of the NMDAR (Brown et al., 2001). However, already suggesting that histamine and spermine might bind to separate sites, histamine, but not spermine, produced a desensitization of recombinant NMDARs (Williams, 1994b). In fact, the effects of histamine and spermine were clearly discriminated in our models. As reported (Woodward and Cueto, 1993), spermine did not enhance NMDA-induced [³H]noradrenaline release from synaptosomes. The potentiation of the same response by *tele*-methylhistamine was neither occluded by spermine, nor antagonized by arcaine. Spermine dramatically increased non-equilibrium [³H]MK-801 binding (Ransom and Stec, 1988; Williams et al., 1989), whereas histamine and *tele*-methylhistamine induced no effect, except an inhibition at high concentrations. Spermine totally inhibited [³H]ifenprodil binding, whereas histamine had again no effect on this binding.

The increase in binding induced by spermine was attenuated by histamine at a concentration without any effect alone. This modulation of spermine effects by histamine, as well as the modulation of histamine effects by polyamines in other systems (Vorobjev et al., 1993; Kuo and Dringenberg, 2008; Watanabe et al., 2008), strongly suggest the existence of allosteric interactions between the histamine and polyamine sites. These observations are reminiscent of those previously made for ifenprodil and polyamine sites. Although they were originally thought to bind at the same site, ifenprodil and polyamines bind in fact to separate sites with allosteric interactions (Kew and Kemp, 1998; Han et al., 2008). Ifenprodil antagonized *tele*-methylhistamine on [³H]noradrenaline release, and decreased histamine-mediated nociception (Watanabe et al., 2008). However, histamine and ifenprodil do not bind at a common site since histamine failed to displace [³H]ifenprodil binding.

JPET # 158543

Therefore, histamine does not bind to the polyamine and ifenprodil sites, but interacts with a distinct histamine site (NMDA_(HA)R). The molecular mechanisms, by which histamine potentiates NMDA, remain unknown and will be better addressed with more potent compounds. The moderate increase of [³H]CGP39653 binding, a radioligand of the glutamate site, induced by *tele*-methylhistamine, might suggest allosteric interactions between the histamine and glutamate sites. The saturating glycine concentration used in patch-clamp confirms a glycine-independent effect of histamine (Bekkers, 1993; Vorobjev et al., 1993; Williams, 1994b; Zwart et al., 1996). In addition, histamine potentiated increases in calcium in the presence of a saturating D-serine concentration. In the release model, the presence of endogenous glycine made it impossible to study adequately the glycine-dependence of the (NMDA_(HA)R). In superfused neurons, the NMDA-induced increase in intracellular calcium strongly suggested that histamine has also a glycine-dependent effect. In the absence of D-serine, NMDA alone had no effect, but histamine and NMDA increased calcium concentrations, suggesting that histamine increased the affinity of the receptor for glycine. Due to perfusion, contaminant glycine is not present at high enough concentrations to serve as co-agonist without histamine, but may become active, when its affinity for the glycine site is increased by histamine. Supporting our data, the potentiation of NMDA currents by histamine was higher at 0.1 μM than at 10 μM glycine, not only in neurons (Vorobjev et al., 1993) but also in transfected oocytes (Williams, 1994b), and on [³H]CGP39653 binding, the effect of *tele*-methylhistamine became non significant with glycine.

The positive effect of its metabolite in the brain suggests that histamine may not be the primary agonist of the NMDA_(HA)R. However, our *in vitro* data show that histamine does potentiate NMDARs in the brain at concentrations (10-100μM) similar to those activating H₁ and H₂ receptors in brain (Brown et al., 2001). Therefore, both endogenous histamine and *tele*-methylhistamine may reach high enough concentrations to activate NMDARs *in vivo*, at

JPET # 158543

least under some conditions. Moreover, activation of the NMDA_(HA)R may have a potential therapeutic interest. A current hypothesis of schizophrenia is based upon a deficit in glutamatergic transmission and predicts that NMDAR activators may constitute a novel class of antipsychotics. Orthosteric agonists are not acceptable in view of their toxicity, and positive allosteric modulators constitute a better approach. Histamine itself was reported to activate the human NMDAR (Munir et al., 1996) However, further work is needed to assess whether the NMDA_(HA)R can become a drug target for the treatment of schizophrenia or other neurological or psychiatric disorder.

JPET # 158543

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JPET # 158543

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Potentiation of NMDA currents by histamine (HA) and *tele*-methylhistamine (*tele*-MeHA) in cultured neurons of rat hippocampus. **A)** Effect of HA and *tele*-MeHA (50 μ M) on currents induced by 50 μ M L-aspartate (asp). **B)** Concentration-response curves of HA and *tele*-MeHA on potentiation of NMDA currents (relative currents).

Figure 2: Characterisation of [³H]noradrenaline release induced by NMDA in rat hippocampal synaptosomes. **A)** NMDA (100 nM - 300 μ M) in the presence of glycine (1 μ M) induces a concentration dependent [³H]noradrenaline release from rat hippocampal synaptosomes. **B)** Effects of various NMDA-receptor antagonists on [³H]noradrenaline release induced by NMDA (200 μ M) and glycine (1 μ M). The antagonists were added at the following final concentrations: Mg²⁺, 1.2 mM; MK-801, 1 μ M; AP5, 20 μ M; 7-KYNA, 100 μ M. Results are means \pm SEM of six determinations from three separate experiments and are expressed as dpm/ μ g protein over spontaneous [³H]noradrenaline release. **p* < 0.001 vs. control NMDA-induced release (one-way ANOVA, Student–Newman–Keuls *post hoc* test).

Figure 3: Potentiation of NMDA-induced [³H]noradrenaline release by histamine derivatives in rat hippocampal synaptosomes. **A)** Effects of L-histidine, histamine, *tele*-methylhistamine (*tele*-MeHA), *pros*-methylhistamine (*pros*-MeHA), and (*R*)-methylhistamine (*(R)*-MeHA) at 100 μ M, on [³H]noradrenaline release induced by NMDA (200 μ M) and glycine (1 μ M). Results are means \pm SEM of 6-10 determinations from 3-5 separate experiments and expressed as dpm/ μ g protein over spontaneous efflux. * *p* < 0.001

JPET # 158543

vs control NMDA-induced release (one-way ANOVA, Student–Newman–Keuls *post hoc* test). **B)** Concentration-response curves of HA and *tele*-MeHA. Each point represents the mean \pm SEM of values obtained in 3-8 separate experiments. **C)** Effects of the NMDA-receptor antagonists ifenprodil (■), MK-801 (○), impromidine (●), and the GABA_A-receptor antagonist bicuculline (Δ) on the potentiation of [³H]noradrenaline release induced by *tele*-MeHA (100μM). Each point represents the mean \pm SEM of values from three experiments expressed as per cent of the effect of *tele*-MeHA.

Figure 4: Characterization of the effect of various histamine derivatives on NMDA-induced [³H]noradrenaline release. **A)** Concentration-response curves of 2-pyridylethylamine (PEA) (Δ), 4-methylimidazole (■), burimamide (□) and (*R*) α -methylhistamine ((*R*) α -MeHA) (○). Each point represents the values obtained in three separate experiments. **B)** Effects of the NMDA-receptor antagonists ifenprodil (■) and impromidine (●) on the potentiation induced by PEA (100μM). Each point represents the values from three separate experiments.

Figure 5: Effects of NMDAR-antagonists on *tele*-MeHA-induced [³H]noradrenaline release. [³H]Noradrenaline release induced by *tele*-MeHA (100μM) added alone from hippocampal synaptosomes was inhibited by the open-channel blocker MK-801, the NR2B selective antagonist ifenprodil and the antagonist of histamine at NMDARs, impromidine. Results are means \pm SEM from six separate determinations and are expressed as dpm /μg protein over spontaneous [³H]noradrenaline release. **p* < 0.001 vs. *tele*-MeHA alone (one-way ANOVA, Student–Newman–Keuls *post hoc* test).

JPET # 158543

Figure 6: Effects of histamine and spermine on [³H]noradrenaline release in rat hippocampus. Synaptosomes were incubated with spermine (10 μM or 1 mM) and/or *tele*-MeHA (200 μM). When required, the polyamine antagonist arcaine was added (1 or 100 μM). Results are means ± SEM from three experiments run in duplicate and are expressed as percent of NMDA-induced release over spontaneous [³H]noradrenaline efflux. * $p < 0.001$ when compared to NMDA-induced release. NS, non-significant when compared to *tele*-MeHA induced release (one-way ANOVA, Student–Newman–Keuls *post hoc* test).

Figure 7: Effects of histamine and spermine on [³H]MK-801 binding to rat hippocampal membranes. (A) Spermine, histamine and *tele*-MeHA were added alone (B). In other sets of experiments, histamine and *tele*-MeHA were tested alone or against spermine. Data are means ± SEM from 3 separate experiments with quadruplicate determinations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control, § $p < 0.001$ vs. spermine alone. (one-way ANOVA, Student–Newman–Keuls *post hoc* test). (C) Effect of histamine (100 μM) on the inhibition curve of ifenprodil. Each point represents the values from quadruplicate determinations.

Figure 8: Effects of histamine and spermine on association kinetics of [³H]MK-801. [³H]MK-801 (2 nM) was incubated from 1 min up to 1 h with 10 μM spermine or 50 μM histamine. Each point represents the mean ± SEM of values from four separate experiments with 3-8 determinations.

Figure 9: Effects of histamine and spermine on [³H]CGP39653 binding, a radioligand of the glutamate site. [³H]CGP39653 (3 nM) was incubated with spermine (10 μM) or *tele*-

JPET # 158543

methylhistamine (50 μM) in the absence (A) or presence (B) of 30 μM glycine. Data are means \pm SEM from three separate experiments with 4-18 determinations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control. (one-way ANOVA, Student–Newman–Keuls *post hoc* test).

Figure 10: Effect of histamine and spermine on [^3H]ifenprodil binding to hippocampal membranes. Histamine or spermine was incubated with 2nM [^3H]ifenprodil in the presence of the sigma ligand GBR 12909 (3 μM). Data are means \pm SEM from 3-5 determinations. *** $p < 0.001$ vs control, (one-way ANOVA, Student–Newman–Keuls *post hoc* test).

Figure 11: Effect of histamine on NMDA-induced increase in intracellular Ca^{2+} in single hippocampal neurons. The horizontal black bars indicate the perfusion periods with the various compounds. Results are expressed as per cent of the increase induced by NMDA (20 μM) and D-serine (10 μM). Each point represents the mean \pm SEM of values obtained in 23 single neurones originating from two independent cultures. * $p < 0.001$ vs NMDA + D-serine (A) or basal concentrations (B) (one-way ANOVA, Student–Newman–Keuls *post hoc* test).

JPET # 158543

TABLE 1

Potencies of various histamine derivatives at NMDAR-mediated responses

	Potentialiation of [³ H]noradrenaline release	Potentialiation of NMDA currents	Property at brain histaminergic receptors
agonists (EC ₅₀ , μM) (relative potency) (intrinsic activity)			
Histamine	99 ± 17 (100) (1)	1.7 -10 ^{a,b,c} (100) (1)	agonist
Histidine	no effect at 100	no effect at 100 ^c	not active
4-Methylimidazole	93 ± 10 (106) (1)	not tested	not active
<i>pro</i> -Methylhistamine	no effect at 100	no effect at 10 ^c	not active
<i>tele</i> -Methylhistamine	97 ± 22 (102) (1)	7.2 ± 1.5 (122) (1)	not active
N ^α -Methylhistamine	no effect at 100	no effect at 10 ^c	agonist
N ^α ,N ^α -Dimethylhistamine	no effect at 100	not tested	agonist
(R)α-Methylhistamine	41 ± 24 (241) (0.3)	EC ₅₀ ≈ 10 ^c (100)	H ₃ agonist
Burimamide	6 ± 2 (1650) (0.4)	not tested	H ₂ /H ₃ antagonist
2-Pyridylethylamine	5 ± 0.5 (1980) (1)	not tested	H ₁ agonist
antagonists/ inverse agonists (IC ₅₀ , μM)			
Impromidine	15 ± 3	≈ 10 ^b	H ₂ agonist/H ₃ antagonist
Imetit	Inhibitory effect at 100	Inhibitory effect at 10 ^c	H ₃ agonist
Thioperamide	no effect at 2	no effect at 10 ^b	H ₃ antagonist

The EC₅₀ values on [³H]noradrenaline release were obtained from the concentration-response curves shown in figures 3 and 4. The relative potencies were determined by comparison to that of histamine (=100). The intrinsic activities were determined by comparison to the maximal effect of histamine.

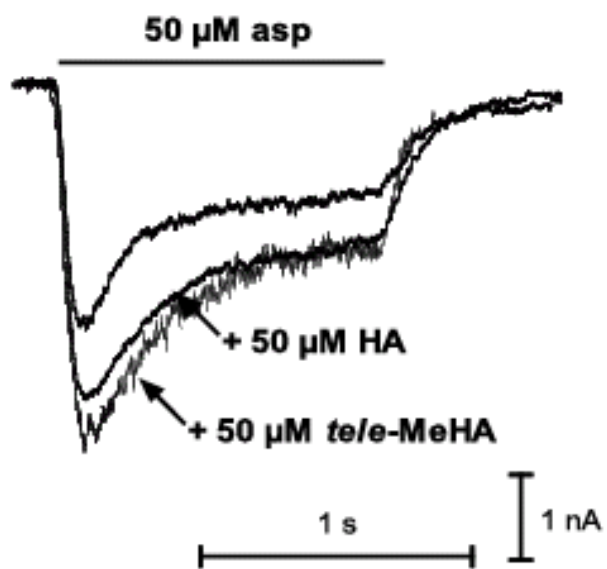
^a Data from (Vorobjev et al., 1993).

^b Data from (Bekkers, 1993).

^c Data from (Williams, 1994a).

Figure 1

A



B

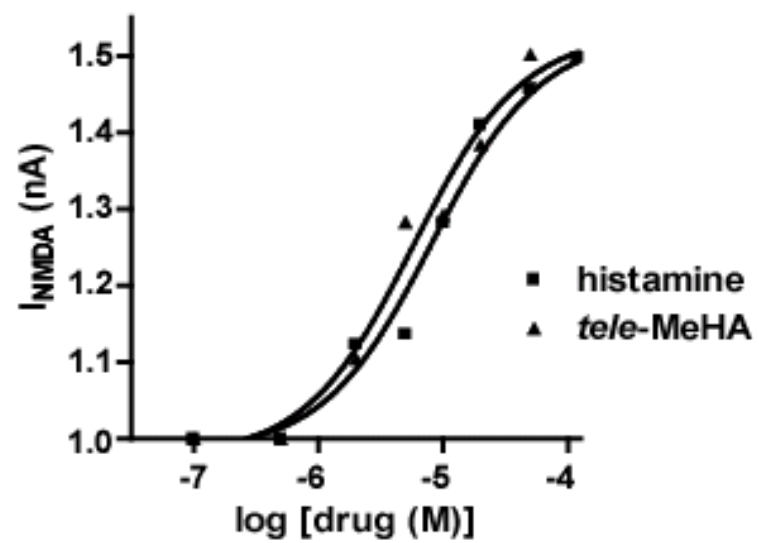
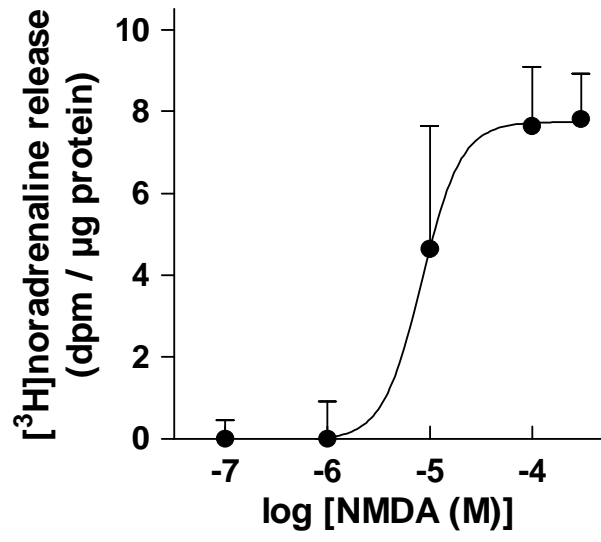


Figure 2

A



B

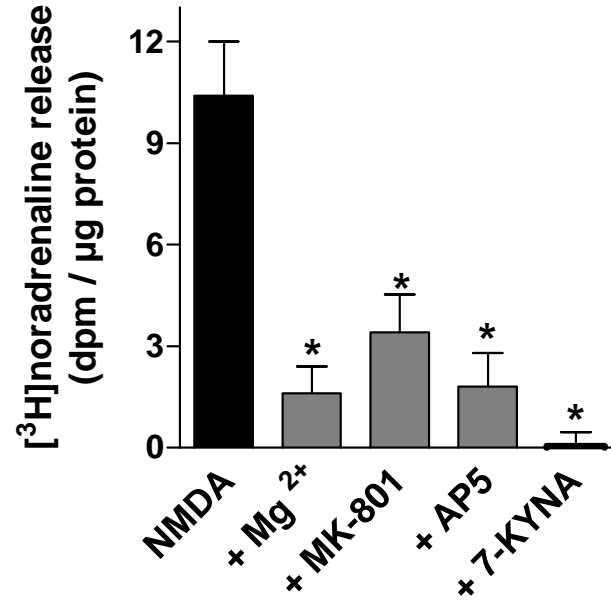


Figure 3

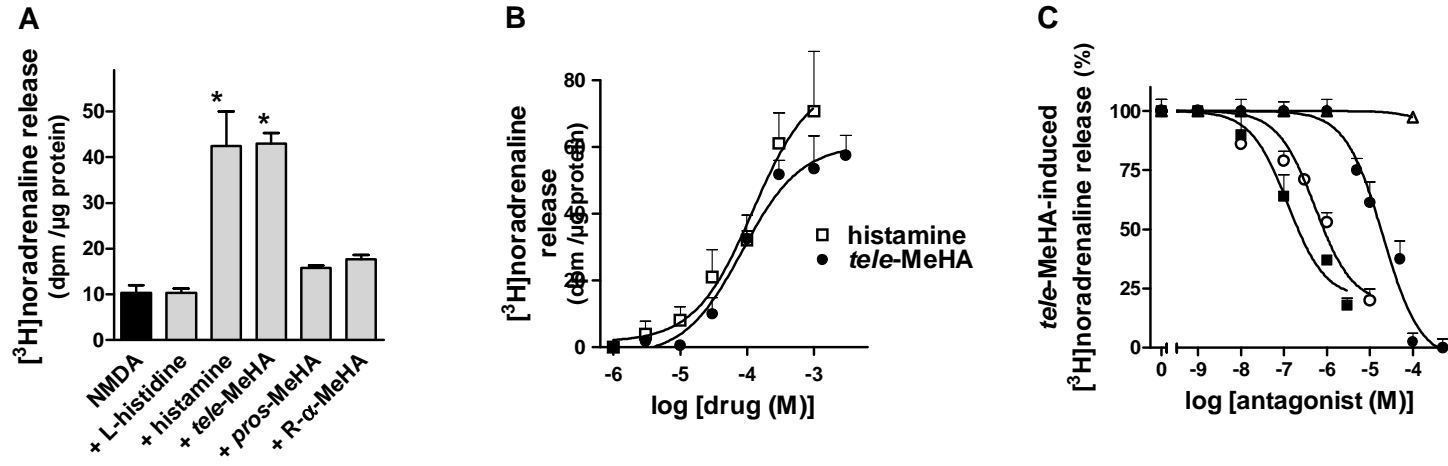


Figure 4

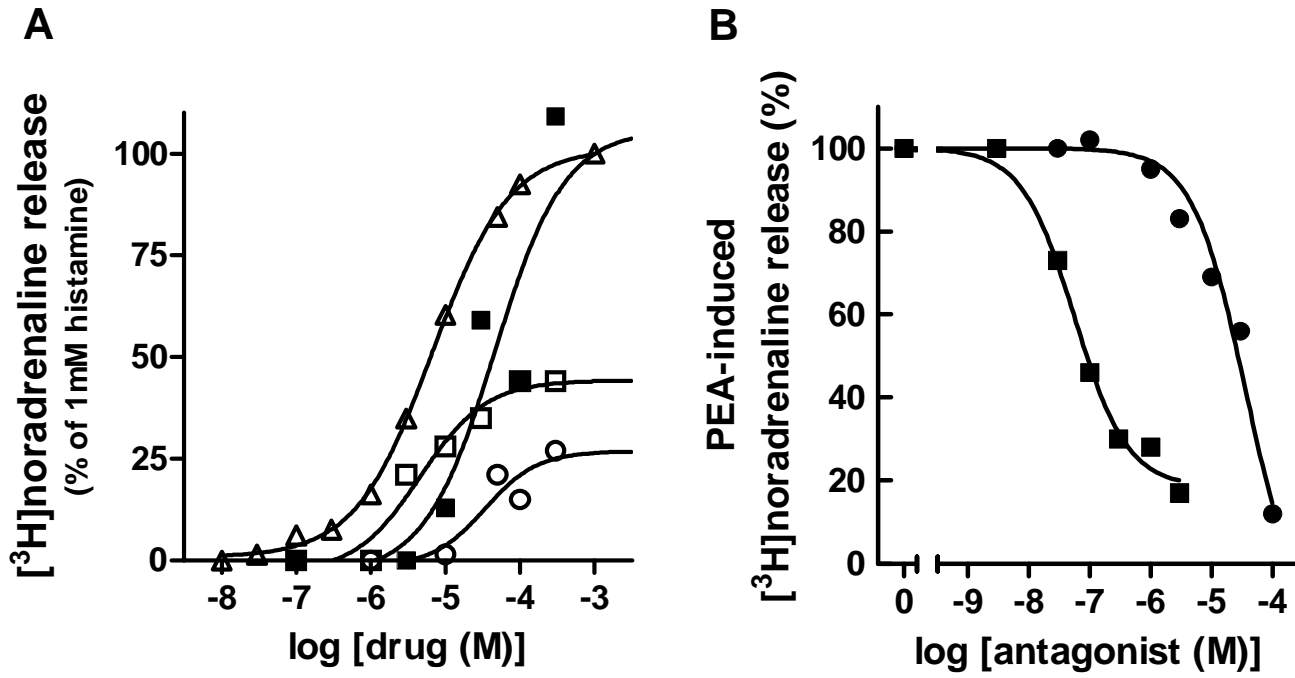


Figure 5

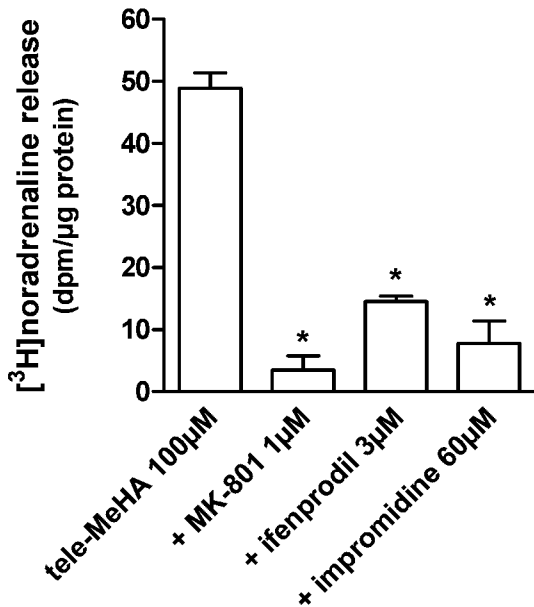
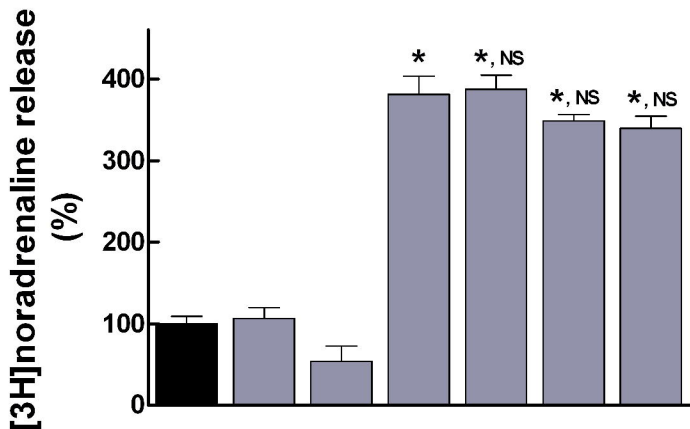


Figure 6



NMDA	+	+	+	+	+	+	+
spermine	-	10 μ M	1 mM	-	-	-	+
<i>tele</i> -MeHA	-	-	-	+	+	+	+
arcaine	-	-	-	-	1 μ M	100 μ M	-

Figure 7

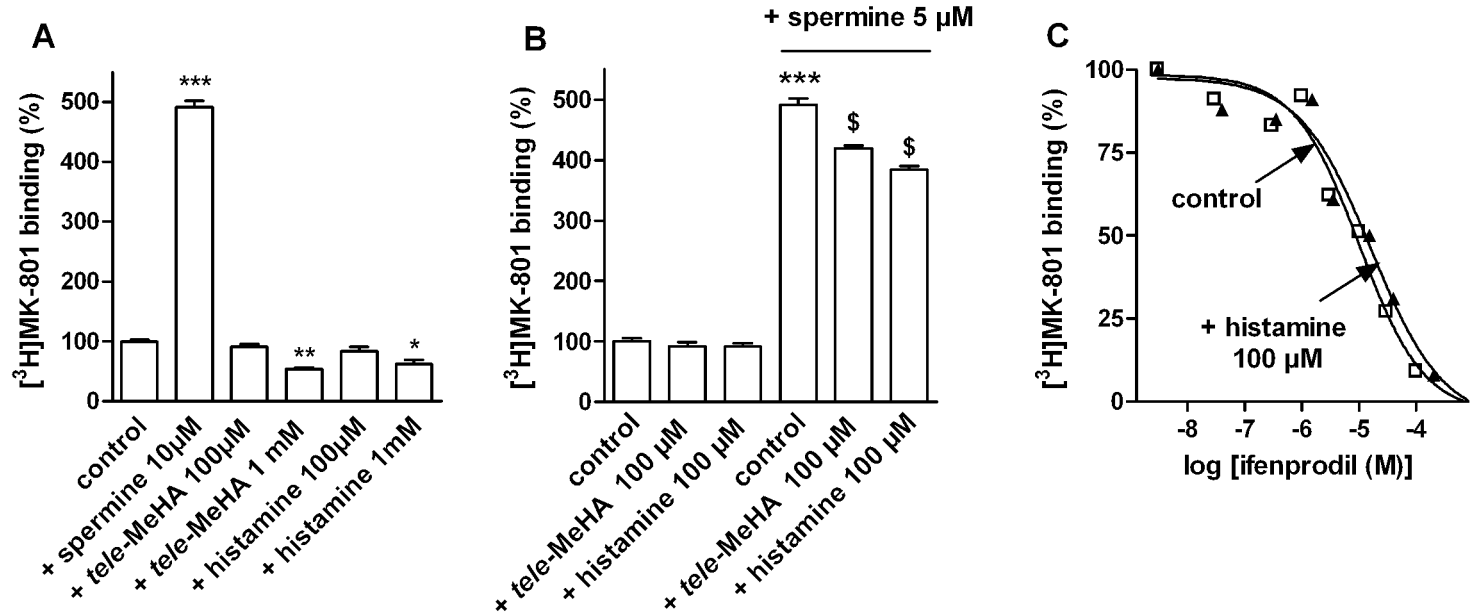


Figure 8

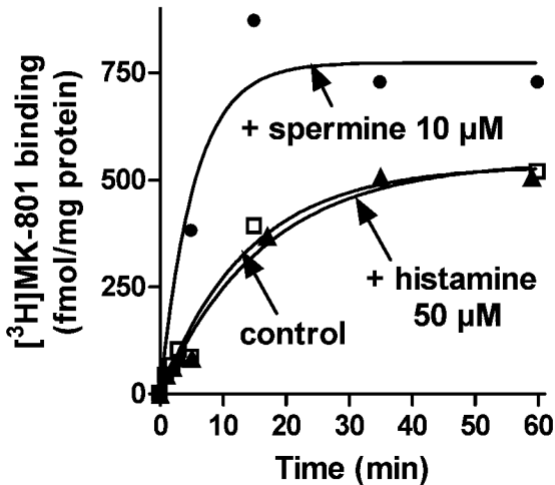


Figure 9

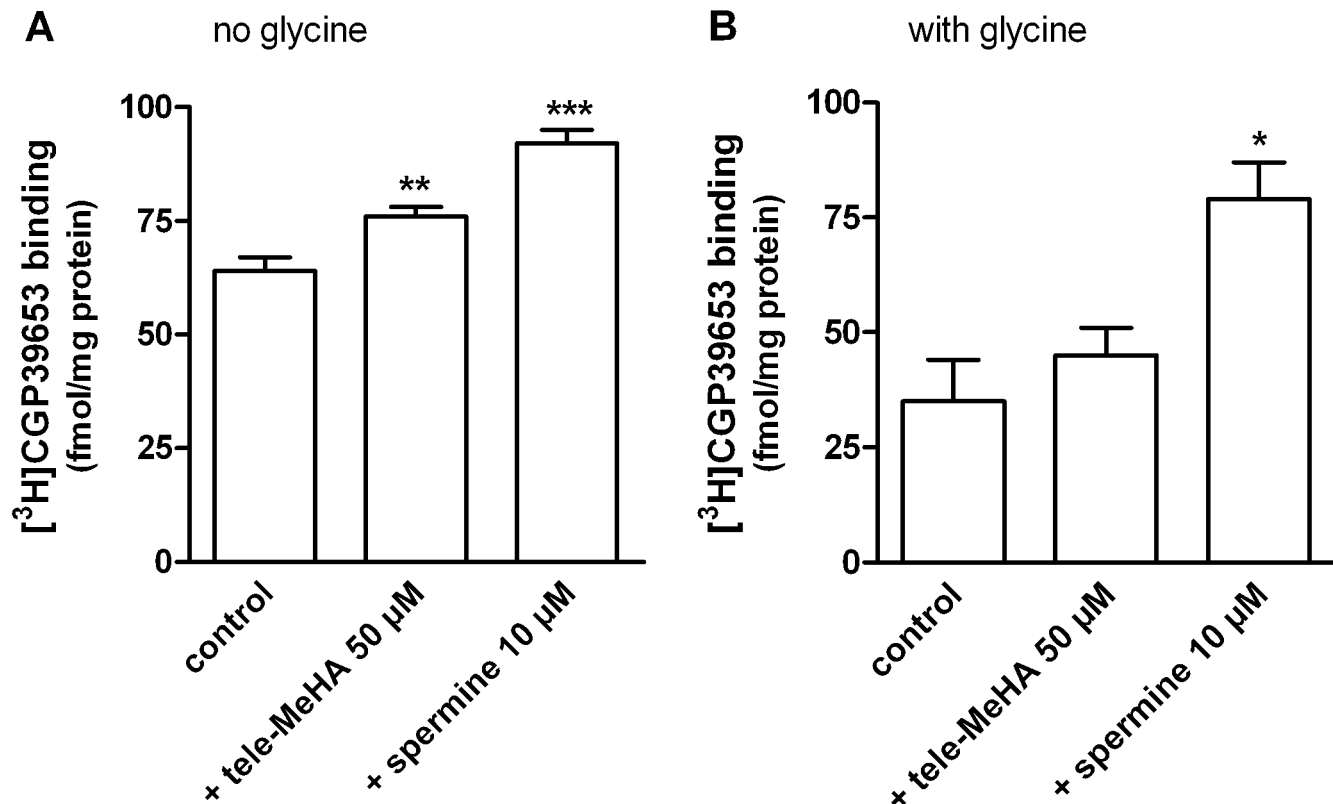


Figure 10 :

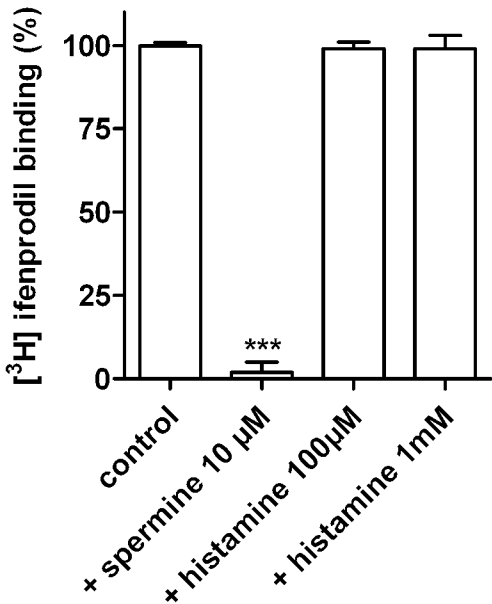


Figure 11

