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RESEARCH PAPER

The histamine autoreceptor is a short isoform of the H_3 receptor

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BACKGROUND AND PURPOSE
The histamine H_3 receptor was identified as the autoreceptor of brain histaminergic neurons. After its cloning, functional H_3 receptor isoforms generated by a deletion in the third intracellular loop were found in the brain. Here, we determined if this autoreceptor was the long or the short isoform.

EXPERIMENTAL APPROACH
We hypothesized that the deletion would affect H_3 receptor stereoselectivity. The effects of the enantiomers of two chiral ligands, N^{α}-methyl-α-chloromethylhistamine (N^{α}Me-αClMeHA) and sopromidine, were investigated on cAMP formation at the $H_{3(445)}$ and $H_{3(413)}$ receptor isoforms, common to all species. They were further compared with their effects at autoreceptors. They were also compared on [^{35}S]GTPγ[S] binding to membranes of rat cerebral cortex, striatum and hypothalamus, the richest area in autoreceptors.

KEY RESULTS
The stereoselectivity of N^{α}Me-αClMeHA enantiomers as agonists was similar at the $H_{3(413)}$ receptor isoform and autoreceptors, but lower at the long isoform. While (S) sopromidine did not discriminate between the isoforms, (R) sopromidine was an antagonist at the $H_{3(413)}$ receptor isoform and autoreceptors, but a full agonist at the long isoform. In rat brain, stereoselectivity of N^{α}Me-αClMeHA was higher in the hypothalamus than in cerebral cortex or striatum, whereas the opposite pattern was found for sopromidine.

CONCLUSIONS AND IMPLICATIONS
The pharmacological profiles of H_3 receptor isoforms differed markedly, showing that the function of autoreceptors was fulfilled by a short isoform, such as the $H_{3(413)}$ receptor. Development of drugs selectively targeting autoreceptors might enhance their therapeutic efficacy and/or decrease incidence of side effects.

Abbreviations
αMeHA, α-methylhistamine; AA, arachidonic acid; IPX, iodoproxyfan; N^{α}Me-αClMeHA, N^{α}-methyl-α-chloromethylhistamine

Introduction

The histamine H_3 receptor was identified as an autoreceptor regulating histaminergic neuron activity in the brain (Arrang *et al.*, 1983; 1987; receptor nomenclature follows Alexander *et al.*, 2011). However, H_3 receptors are also present on many other neuronal populations (Pillot *et al.*, 2002), either as post-synaptic receptors or as heteroreceptors (Schlicker *et al.*, 1994). The coupling of the H_3 receptor to $G_{i/o}$ proteins was confirmed by its cloning in humans (Lovenberg *et al.*, 1999). Activation of recombinant H_3 receptors inhibits adenylate cyclase, assayed as cAMP accumulation (Lovenberg *et al.*,

1999), and activates phospholipase A_2, assessed as arachidonic acid (AA) release (Morisset *et al.*, 2000).

Various recombinant isoforms of the H_3 receptor have been identified in different species including humans. These isoforms are generated by the deletion of a pseudo-intron, variable in length, located in the third intracellular loop of the receptor (Hancock *et al.*, 2003). They are all expressed in the brain, but their respective functions therein remain unknown. Binding studies on these H_3 receptor isoforms revealed only moderate pharmacological differences (Coge *et al.*, 2001; Morisset *et al.*, 2001; Rouleau *et al.*, 2004). Functional studies with standard agonists revealed some differences in coupling between rat isoforms, which, however, were dependent on the response studied and cell type used (Morisset *et al.*, 2000; Drutel *et al.*, 2001). These agonists also displayed a higher potency at the human $H_{3(365)}$ receptor than at the human $H_{3(445)}$ receptor (Wellendorph *et al.*, 2002; Bongers *et al.*, 2007). Using various chiral histamine derivatives, we have previously shown a strong stereoselectivity of the rat H_3 autoreceptor on inhibition of histamine release, with a preference of the H_3 receptor for (+) enantiomers (i.e. corresponding to the S-configuration of L-histidine), such as R-(α)-methylhistamine (R-αMeHA) (Arrang *et al.*, 1985a). In the present study, we tried to take advantage of this stereoselectivity to identify which of the long or short isoforms function as the autoreceptors. Our hypothesis was that the deletion within the third intracellular loop of the H_3 receptor would generate three-dimensional modifications recognized by chiral compounds. We tested this hypothesis by characterizing the effects of N^{α}-methyl-α-chloromethylhistamine (N^{α}Me-αClMeHA) and sopromidine, two chiral ligands for which the H_3 receptor is stereoselective (Arrang *et al.*, 1985a), at the rat long (non-deleted) $H_{3(445)}$ isoform and short $H_{3(413)}$ isoform. This short isoform was selected for the study because, in contrast to the others, it is the only one to be maintained in the brain from all species including rat (Morisset *et al.*, 2001), mouse (Rouleau *et al.*, 2004), guinea pig (Tardivel-Lacombe *et al.*, 2000), monkey (Strakhova *et al.*, 2008) and human (Coge *et al.*, 2001; Tardivel-Lacombe *et al.*, 2001). The stereoselectivity of each of the two isoforms was compared with the autoreceptor. Moreover, we used H_3 receptor-mediated [^{35}S]GTPγ[S] binding to rat brain membranes (Rouleau *et al.*, 2002) in order to further compare the effects of N^{α}Me-αClMeHA and sopromidine isomers in the hypothalamus, a region known to contain a very high density of histaminergic fibres and, hence, of H_3 autoreceptors (Panula and Airaksinen, 1991; Tohyama *et al.*, 1991; Wouterlood and Steinbusch, 1991), as well as in the cerebral cortex and striatum. All these approaches showed marked differences between isoforms and revealed that the autoreceptor is a short isoform.

Methods

Cloning and expression of the $rH_{3(445)}$ and $rH_{3(413)}$ receptor isoforms

The two rat H_3 (rH_3) receptor isoforms were cloned and expressed as previously described (Morisset *et al.*, 2001). Briefly, cDNA inserts corresponding to the full-length coding sequence of the rat H_3 isoforms, $rH_{3(445)}$ or $rH_{3(413)}$, were ligated into the mammalian expression vector pCIneo (Promega, Charbonnières, France). CHO-K1 cells were transfected with SuperFect Reagent (Qiagen, Courtaboeuf, France). Stable transfectants were selected with 2 mg·mL^{-1} of Geneticin, tested for their expression level of [^{125}I]iodoproxyfan (IPX) binding sites and maintained in the presence of 1 mg·mL^{-1} of Geneticin.

[^{125}I]IPX binding assays

CHO cells transfected with the two isoforms, CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells, and expressing 400–500 fmol·mg^{-1} protein were harvested, homogenized in ice-cold phosphate buffer (50 mM Na_2HPO_4/KH_2PO_4, pH 7.5) and centrifuged (140× *g* for 10 min at 4°C). The pellets were then suspended in 10 mL of phosphate buffer and homogenized with a Polytron homogenizer (Polytron, Inc., Norcross, GA, USA). After centrifugation at 23 000× *g* for 30 min at 4°C, the last pellets were washed superficially and sonicated for 30 s in fresh ice-cold buffer. Binding assays were performed as described previously (Morisset *et al.*, 2001). Briefly, aliquots of membrane suspensions (≈20 µg of protein) were incubated for 60 min at 25°C with 30 pM [^{125}I]IPX alone or together with drugs in increasing concentrations (200 µL final volume). Non-specific binding was determined using the selective H_3 receptor agonist imetit at 1 µM. Incubations, performed in triplicate, were stopped by rapid filtration through glass microfibre filters (GF/B; Whatman, Clifton, NJ, USA) presoaked in 0.3% polyethylenimine. Radioactivity trapped on filters was counted with a gamma counter.

[^{3}H]AA release

CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells expressing 400–500 fmol·mg^{-1} protein were seeded 24 h before the assay in 24 well-plates. After incubation for 2 h at 37°C with 0.5 µCi [^{3}H]AA in DMEM-Nut mix F-12 (Invitrogen Life Technology, Cergy Pontoise, France) containing 0.2% BSA, the cells were washed twice and the drugs tested [histamine, R-αMeHA and for CHO($rH_{3(413)}$R) cells, S-(α)-methylhistamine] were added in increasing concentrations and incubated for 10 min. Cells were then incubated for 30 min with 2 µM of the Ca^{2+} ionophore A23187. [^{3}H]AA release was determined by liquid scintillation counting.

cAMP accumulation

CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells were seeded 24 h before the assay in 96 well-plates. After incubation for 10 min at 37°C with 3 µM forskolin, drugs [histamine, R-αMeHA, R and S enantiomers of N^{α}Me-αClMeHA and of sopromidine] were added, when required, at increasing concentrations in DMEM-Nut mix F-12 containing 100 µM isobutylmethyl xanthine. cAMP was extracted and measured by radioimmunoassay according to the instructions of the manufacturer (PerkinElmer Life Sciences, Boston, MA, USA).

[^{35}S]GTPγ[S] binding assays

[^{35}S]GTPγ[S] binding to brain membranes was performed as previously described (Rouleau *et al.*, 2002). The cerebral cortex, striatum and hypothalamus were dissected out from brains of male Wistar rats (160–200 g, Janvier, Le Genest-

Saint-Isle, France), homogenized in ice-cold buffer (50 mM Tris/HCl, pH 7.4) and centrifuged (140× *g* for 10 min at 4°C). The supernatants were centrifuged twice at 23 000× *g* for 15 min at 4°C. The final pellets were suspended in 50 volumes of buffer. Membranes (8–22 μg) were pretreated for 30 min at 25°C with adenosine deaminase (1 U·mL^{-1}; Roche, Meylan, France) and incubated for 60 min at 25°C with 0.1 nM [^{35}S]GTPγ[S] and, when required, the various drugs were tested (R and S enantiomers of N$^{\alpha}$Me-αClMeHA and of sopromidine), in 1 mL of assay buffer (50 mM Tris/HCl, 50 mM NaCl, 5 mM $MgCl_2$, 10 μM GDP, 0.02% BSA, pH 7.4). In order to prevent any interaction of the drugs with histamine H_1 and H_2 receptors, all incubations were performed in the presence of 100 nM mepyramine and 10 μM cimetidine. The non-specific binding was determined using GTPγS (10 μM). Incubations were stopped by rapid filtration under vacuum through Whatman GF/B filters. Filters were washed twice with 4 mL of ice-cold water and the radioactivity retained on the filters was measured by liquid scintillation spectrometry.

Analysis of data

The curves were analysed with an iterative least-squares method by non-linear regression using a one-site cooperative model (Gbahou *et al.*, 2006). The method provided estimates for EC_{50} values, IC_{50} values and their SEM. The apparent affinity constants (K_i values) of N$^{\alpha}$Me-αClMeHA enantiomers on inhibition of [^{125}I]IPX binding were calculated from their IC_{50} values by using the relationship (Cheng and Prussoff, 1973): $K_i = IC_{50}/1 + (S/X)$, where S represents the concentration (30 pM) and X is the apparent dissociation constant (K_D) of [^{125}I]IPX at the $rH_{3(445)}$ receptor isoform (85 pM) and at the $rH_{3(413)}$ receptor isoform (82 pM). The same relationship was used to calculate K_i values of sopromidine isomers tested against histamine (100 nM) on cAMP formation at recombinant isoforms. In that case, the total curves were analysed with X representing the EC_{50} values of histamine at the $rH_{3(445)}$ (9 nM) and $rH_{3(413)}$ receptor isoforms (15 nM). Their K_i values on specific [^{35}S]GTPγ[S] binding induced by 1 μM imetit to brain membranes were also calculated with the same relationship, taking into account only the antagonistic part of the curves (above 100%) and an EC_{50} value of imetit of 2 ± 1 nM (not shown).

Statistical evaluation of the results was performed by one-way ANOVA, followed by Student's Newman–Keuls *post hoc* test. For statistical comparison of the EC_{50} values, IC_{50} values or maximal effects of two compounds (i.e. two enantiomers or R-αMeHA vs. histamine), the two corresponding curves were analysed by two-way ANOVA, followed by Bonferroni *post hoc* test.

The activity ratio of two enantiomers (termed S/R ratio when the S enantiomer was preferred and R/S ratio when the R enantiomer was preferred) was determined as follows: [EC_{50} (or K_i) value of the preferred (R or S) enantiomer/EC_{50} (or K_i) value of the non-preferred (R or S) enantiomer] and was used as an index of the stereoselectivity (Arrang *et al.*, 1985a).

Materials

[^{125}I]IPX (2000 Ci·mmol^{-1}) was prepared as described (Krause *et al.*, 1997). [^{3}H]AA (211 Ci·mmol^{-1}) and [^{35}S]GTPγ[S] (1250 Ci·mmol^{-1}) were from PerkinElmer Life Sciences. Histamine, imetit, thioperamide, isobutylmethyl xanthine, forskolin and BSA were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Ionophore A23187 and adenosine deaminase were obtained from Roche. The enantiomers of α-MeHA, N$^{\alpha}$Me-αClMeHA and sopromidine were provided by W. Schunack (Freie Universität Berlin, Germany).

Results

Effect of histamine and R-α-methylhistamine on forskolin-induced cAMP accumulation and A23187-evoked [^{3}H]AA release in CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells

The cloning of the rat receptor isoforms $rH_{3(445)}$ or $rH_{3(413)}$ in CHO cells yielded numerous clones stably expressing various densities of these isoforms. As expected from the negative coupling of the H_3 receptor to adenylate cyclase (Lovenberg *et al.*, 1999; Morisset *et al.*, 2000), histamine used at a maximal concentration (1 μM) induced an inhibition of forskolin-induced cAMP accumulation, which was already significant at a density as low as ~20 fmol·mg^{-1} protein of $rH_{3(445)}$ receptor (Figure 1). The magnitude of this inhibition dramatically increased with the density of each isoform, to become almost total at a density of 1000 fmol·mg^{-1} protein of $rH_{3(413)}$ receptor (cAMP rate of 299 ± 59 fmol vs. 2788 ± 198 fmol with forskolin alone, Figure 1). The influence of the receptor density on the histamine effect was similar for both isoforms. At a density of 300 fmol·mg^{-1} protein, histamine inhibited forskolin-induced cAMP accumulation by 45 ± 5% and 45 ± 10% with the receptor isoforms $rH_{3(445)}$ and $rH_{3(413)}$ respectively. At a density of 400–500 fmol·mg^{-1} protein, the histamine-induced inhibition was 70 ± 3% and 74 ± 3% respectively (Figure 1).

These cell lines expressing 400–500 fmol·mg^{-1} protein of each isoform were selected in subsequent experiments. The agonist effect of histamine displayed a similar profile at $rH_{3(445)}$ and $rH_{3(413)}$ isoforms, with a maximal inhibition of cAMP formation of 75–80% and EC_{50} values of 9 ± 2 and 15 ± 4 nM respectively (Figure 2A and Table 1). The H_3 receptor agonist R-αMeHA behaved as a full agonist with EC_{50} values of 3.6 ± 3.4 nM at $rH_{3(445)}$ and 1.8 ± 0.2 nM at $rH_{3(413)}$ receptors (Figure 2A), leading to potencies of R-αMeHA relative to histamine at the two isoforms of 266% and 833% respectively. Two-way ANOVA indicated that the difference between the curves of R-α-MeHA and histamine was significant for $rH_{3(413)}$ receptors [$F(1,36) = 19.74$, $P < 0.0001$], but not for $rH_{3(445)}$ receptors [$F(1,14) = 2.54$, $P = 0.13$]. *Post hoc* analysis confirmed that R-α-MeHA was significantly more potent than histamine at $rH_{3(413)}$ receptors ($P < 0.01$ at 3 nM and $P < 0.05$ at 10 nM, Figure 2A).

On [^{3}H]AA release, a signalling pathway positively coupled to recombinant H_3 receptors (Morisset *et al.*, 2000; Gbahou *et al.*, 2003), histamine increased A23187-evoked [^{3}H]AA release with EC_{50} values of 177 ± 68 and 213 ± 50 nM at $rH_{3(445)}$ and $rH_{3(413)}$ receptor isoforms respectively (Figure 2B). R-αMeHA increased [^{3}H]AA release with EC_{50} values of 1.6 ± 0.9 nM at $rH_{3(445)}$ and 19 ± 5 nM at $rH_{3(413)}$ receptors (Figure 2B), leading to potencies of R-αMeHA rela-

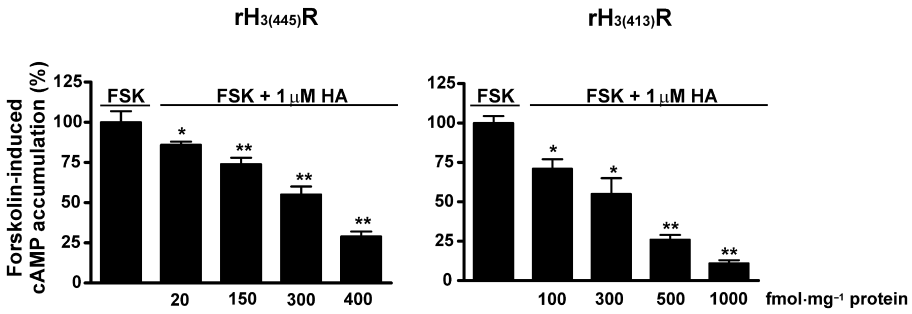

Figure 1

Effect of histamine (HA) on forskolin (FSK)-induced cAMP accumulation in CHO cells expressing various densities of the $rH_{3(445)}$ and $rH_{3(413)}$ receptor isoforms. cAMP accumulation was induced by forskolin (3 μM) alone or in the presence of histamine (1 μM) in CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells expressing increasing isoform densities (up to 1000 fmol·mg^{-1} protein, as determined using [^{125}I]IPX binding assay). The results are means ± SEM of values from two to six different experiments with five determinations each. *$P < 0.01$, **$P < 0.001$, significantly different from forskolin alone.

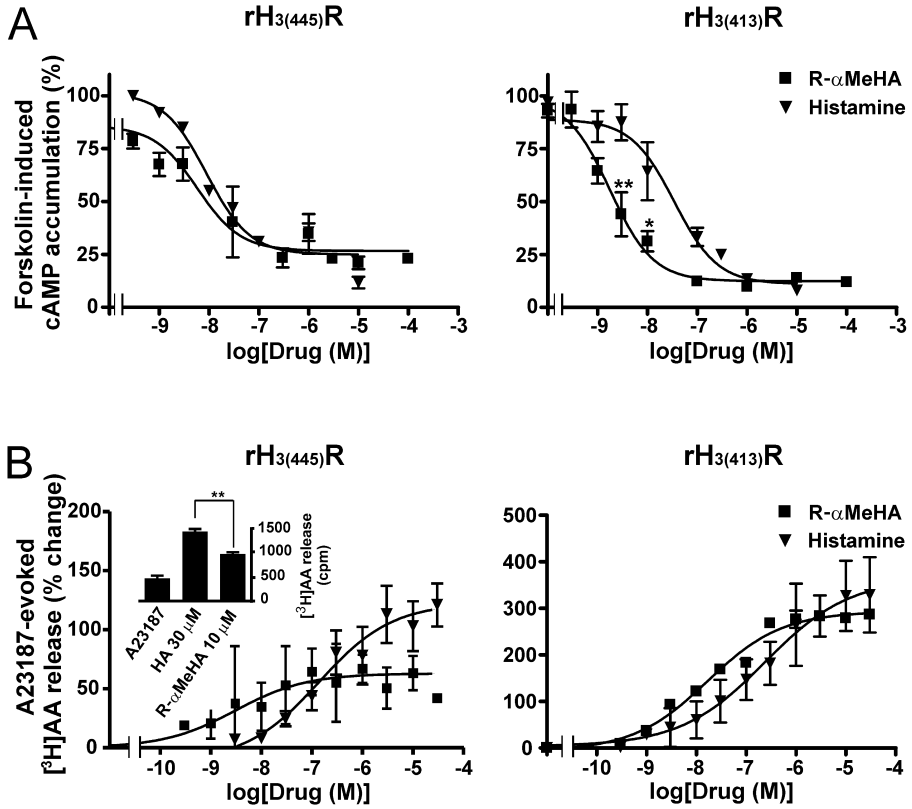

Figure 2

Effects of R-αMeHA and histamine on forskolin-induced cAMP accumulation (A) and A23187-evoked [^{3}H]AA release (B) in CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells expressing 350–500 fmol·mg^{-1} protein of receptor. Results are means ± SEM of values from two to eight separate experiments with three to five determinations each. In (B), the inset represents the effects of both compounds at a maximal concentration within one of the experiments. *$P < 0.05$, **$P < 0.01$, significantly different from histamine.

tive to histamine at the two isoforms of 11063% and 1121% respectively. Two-way ANOVA failed to show any significant difference between the curves of R-α-MeHA and histamine not only at $rH_{3(413)}$ receptors but also at $rH_{3(445)}$ receptors [$F(1,41) = 0.94$, $P = 0.33$]. This finding probably resulted from the variability of the response between experiments coupled to the low magnitude of the increase induced by R-α-MeHA at $rH_{3(445)}$ receptors. However, the curve fitting showed that R-αMeHA behaved as a full agonist at $rH_{3(413)}$ receptors, but as a partial agonist at $rH_{3(445)}$ receptors with an intrinsic activity

Table 1

Compared agonist potencies (EC_{50}, μM) of R(–) and S(+) N^{α}Me-αClMeHA at the recombinant $rH_{3(445)}$ and $rH_{3(413)}$ receptor isoforms and at the rat H_3 autoreceptor

Compounds	$rH_{3(445)}$ receptor	$rH_{3(413)}$ receptor	H_3 autoreceptor[a]
Histamine	0.009 ± 0.002 (100)	0.015 ± 0.004 (100)	0.062 ± 0.014 (100)
R(–) N^{α}Me-αClMeHA	0.27 ± 0.04 (3.3)	81 ± 24 (0.02)	1100 ± 0.5 (0.006)
S(+) N^{α}Me-αClMeHA	0.084 ± 0.014 (11)	1.5 ± 0.8 (1)	5.5 ± 1.9 (1.1)
Ratio S/R	3.2	54	200

[a]Values from Arrang *et al.* (1985a). The values at the two isoforms are derived from the data shown in Figure 3. The potencies relative to histamine (=100) are indicated between parentheses and were calculated as the ratio: EC_{50} value of histamine/EC_{50} value of agonist × 100.

of 50% that of histamine (Figure 2B). In agreement, when the maximal effect of R-α-MeHA was compared with that of histamine within each of the eight experiments performed at $rH_{3(445)}$ receptors with three to five determinations, it was found to be significantly lower ($P < 0.01$) in all the experiments (inset of Figure 2B), with an intrinsic activity of R-αMeHA ranging from 33% to 61% that of histamine, confirming the partial agonism by R-αMeHA at the long isoform (Figure 2B). Due to the limited availability of the drug, the S enantiomer of αMeHA could be tested only at the shorter isoform on [^{3}H]AA release and increased this release with an EC_{50} of 900 ± 450 nM (data not shown). At this $rH_{3(413)}$ isoform, the comparison with histamine yielded therefore a relative potency for S-αMeHA of 24% and the comparison with R-αMeHA yielded a marked stereoselectivity with an activity ratio R/S = 47.4.

Effects of (R) and (S) N^{α}Me-αClMeHA on specific [^{125}I]IPX binding and forskolin-induced cAMP accumulation in CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells

In addition to αMeHA, the effect of N^{α}Me-αClMeHA, another chiral analogue of histamine previously studied at autoreceptors (Arrang *et al.*, 1985a), was investigated at $rH_{3(445)}$ and $rH_{3(413)}$ receptor isoforms.

Its two enantiomers were first studied on the [^{125}I]IPX specific binding assay using membranes of CHO cells expressing a similar density of $rH_{3(445)}$ and $rH_{3(413)}$ receptors. The R and S enantiomers of N^{α}Me-αClMeHA inhibited [^{125}I]IPX binding with deduced K_i values at $rH_{3(445)}$ receptors of 480 ± 60 and 220 ± 40 nM respectively (S/R ratio = 2.2). The corresponding K_i values of the R and S enantiomers at $rH_{3(413)}$ receptors were 1.5 ± 0.3 and 1.1 ± 0.2 μM (S/R ratio = 1.4) (Figure 3A).

On cAMP accumulation induced by forskolin, the comparison of their plateau with that of histamine showed that R(–) and S(+) N^{α}Me-αClMeHA behaved as partial agonists at $rH_{3(445)}$ receptors with an intrinsic activity of ~60% that of histamine, but as full agonists at $rH_{3(413)}$ receptors (Figure 3B). At the $rH_{3(445)}$ receptor isoform, their respective EC_{50} values were 270 ± 40 and 84 ± 14 nM, leading to respective potencies relative to histamine of 3.3% and 11% and to an S/R ratio of 3.2 (Figure 3B and Table 1). At the $rH_{3(413)}$ receptor isoform, the respective EC_{50} values of R(–) and S(+) N^{α}Me-αClMeHA were 81 ± 24 and 1.5 ± 0.8 μM, leading to respective potencies relative to histamine of 0.02% and 1% and to an S/R ratio of 54 (Figure 3B and Table 1). The statistical analysis using two-way ANOVA showed a significant difference between the curves of the two enantiomers both at $rH_{3(445)}$ receptors [$F(1,37) = 7.68$, $P < 0.01$] and at $rH_{3(413)}$ receptors [$F(1,37) = 31.54$, $P < 0.0001$]. However, *post hoc* analysis led to significant differences at several concentrations only at $rH_{3(413)}$ receptors ($P < 0.01$ at 1 μM; $P < 0.05$ at 10 and 30 μM, Figure 3B). These data show that if the S(+) enantiomer was preferred to the R(–) enantiomer at both isoforms, the stereoselectivity was more pronounced at the $rH_{3(413)}$ receptors isoform (S/R ratio of 54 vs. 3.2) (Table 1). Moreover, the comparison of the potencies relative to histamine obtained at the two recombinant isoforms with those previously reported at autoreceptors (Arrang *et al.*, 1985a) shows that the relative potency of S(+) N^{α}Me-αClMeHA at $rH_{3(445)}$ receptors (11%) was only 10-fold lower at $rH_{3(413)}$ receptors and at autoreceptors (1% and 1.1%, respectively), whereas the relative potency of R(–) N^{α}Me-αClMeHA at $rH_{3(445)}$ receptors (3.3%) was 200- to 500-fold lower at $rH_{3(413)}$ receptors and autoreceptors (0.02% and 0.006%, respectively), yielding a similar stereoselectivity on these two systems (S/R ratio = 54 vs. 200) (Table 1).

Effects of the R(–) and S(+) enantiomers of sopromidine on forskolin-induced cAMP accumulation in CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells

R(–) and S(+) sopromidine were first investigated alone on forskolin-induced cAMP accumulation (Figure 4A). At the $rH_{3(445)}$ receptor isoform, R(–) sopromidine decreased the response in a concentration-dependent manner (EC_{50} = 79 ± 34 nM), with a maximal effect similar to that of histamine and was therefore behaving as a full agonist at this isoform. In contrast, S(+) sopromidine alone mimicked the effect of thioperamide and increased the response with an EC_{50} of 180 ± 90 nM, thereby acting as an inverse agonist (Figure 4A and Table 2). At the $rH_{3(413)}$ receptor isoform, R(–) sopromidine behaved again as an agonist (EC_{50} = 91 ± 78 nM), but the comparison of its maximal effect with that of histamine indicated that it acted as a very partial agonist with an intrinsic activity of ~20% (Figure 4A). S(+) sopromidine alone behaved also as an inverse agonist at this isoform, with an EC_{50} of 340 ± 92 nM and a maximal effect similar to that of thioperamide.

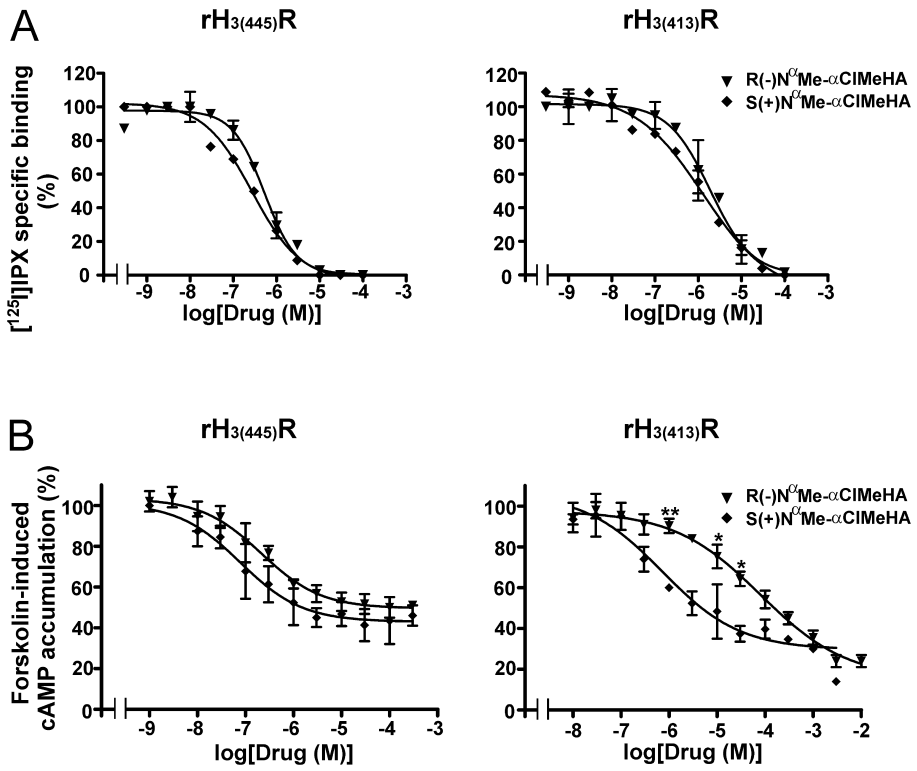

Figure 3

Effects of the R(–) and S(+) enantiomers of N$^{\alpha}$Me-αClMeHA on specific [^{125}I]IPX binding (A) and forskolin-induced cAMP accumulation (B) in CHO($rH_{3(445)}$R) and CHO($rH_{3(413)}$R) cells expressing 500 fmol·mg^{-1} protein of receptor. Each point represents the mean ± SEM of values from two to three different experiments with three to five determinations each. *P < 0.05, **P < 0.01, significantly different from S(+) N$^{\alpha}$Me-αClMeHA.

The antagonist potency of the two enantiomers was then evaluated against a sub-maximal concentration of histamine (100 nM) (Figure 4B). At the $rH_{3(445)}$ receptor isoform, R(–) sopromidine tested up to 300 μM had no apparent effect against histamine. In contrast, the S enantiomer completely reversed the inhibition of forskolin-induced cAMP formation induced by histamine and an enhancement of the response was even observed at the highest concentration due to its own inverse agonist effect. Analysis of the total curve yielded a K_i value for S(+) sopromidine of 250 ± 120 nM (Figure 4B and Table 2). At the $rH_{3(413)}$ receptor isoform, R(–) sopromidine partially reversed the effect of histamine with a K_i of 63 ± 40 nM and a plateau similar to that obtained with the compound added alone (Figure 4). The S enantiomer completely reversed the effect of histamine with a K_i value of 220 ± 100 nM. Again, its own inverse agonist effect enhanced the response at the highest concentration (Figure 4B and Table 2). Two-way ANOVA confirmed the statistical significance of the difference between the two curves [$F(1,34)$ = 12.39, P < 0.001] and *post hoc* analysis confirmed the significant difference of the plateau (P < 0.01 at 300 μM; P < 0.05 at 30 μM, Figure 4B). The comparison of the properties of R(–) and S(+) sopromidine at the two recombinant isoforms with those previously reported at autoreceptors (Arrang *et al.*, 1985a) shows that R(–) sopromidine behaves as a full agonist at the $rH_{3(445)}$ receptor isoform, but as an antagonist with a similar potency at the $rH_{3(413)}$ receptor isoform and at autoreceptors (K_i of 63 ± 40 and 56 ± 22 nM, respectively, Table 2). In contrast, S(+) sopromidine behaved as an antagonist/inverse agonist in the three systems (Table 2).

Effects of the R(–) and S(+) enantiomers of N$^{\alpha}$Me-αClMeHA and sopromidine on [^{35}S]GTPγ[S] binding to rat brain membranes

The effects of the enantiomers of N$^{\alpha}$Me-αClMeHA and sopromidine were studied at native H_3 receptors mediating [^{35}S]GTPγ[S] binding to rat brain membranes (Rouleau *et al.*, 2002). In [^{35}S]GTPγ[S] binding assays, the H_3 selectivity of their effects was ensured by blockade of H_1 and H_2 receptors with corresponding antagonists, that is, mepyramine and cimetidine, used at maximal concentrations. R(–) and S(+) N$^{\alpha}$Me-αClMeHA increased specific [^{35}S]GTPγ[S] binding in the cerebral cortex, striatum and hypothalamus in a concentration-dependent and saturable manner. The EC_{50} value of the S enantiomer was similar in the three regions (3.8 ± 0.4, 9.2 ± 3.1 and 6.1 ± 5.8 μM in the cerebral cortex, striatum and hypothalamus respectively). The EC_{50} value of the R enantiomer increased from 9.7 ± 1.9 μM in the striatum to 14 ± 7 μM in the cerebral cortex and to 77 ± 34 μM in the hypothalamus, leading S/R ratios to increase from 1.0 in the striatum to 3.7 in the cerebral cortex and 13 in the hypothalamus (Figure 5A). The plateau reached by the two enantiomers was not significantly different from the plateau reached by imetit in the cerebral cortex and striatum (116 ± 1.6% and 118 ± 3.5%, respectively), indicating that the compounds were acting as full agonists in these two regions.

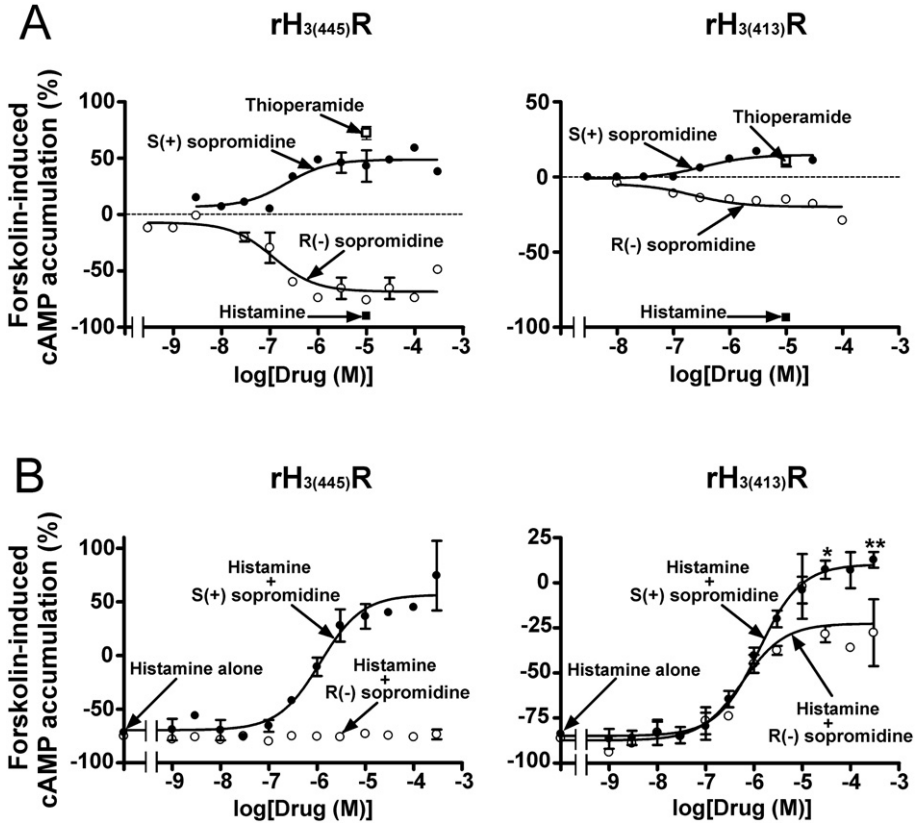

Figure 4

Effects of the R(–) and S(+) enantiomers of sopromidine on forskolin-induced cAMP accumulation in CHO($rH_{3(445)}R$) and CHO($rH_{3(413)}R$) cells. Cells were incubated with 3 μM forskolin and, when required, increasing concentrations of R(–) or S(+) sopromidine added alone (A) or in the presence of 100 nM histamine (B). The data are the means ± SEM of values from two to four separate experiments with five determinations each. *$P < 0.05$, **$P < 0.01$, significantly different from histamine + R(–) sopromidine.

However, in the hypothalamus, their maximal effect was half of that of imetit (3 ± 1% and 4 ± 1% vs. 8 ± 2%, respectively), showing that they behaved as partial agonists in this region. In this area, two-way ANOVA indicated a difference between the two curves close to statistical significance [$F(1,52) = 3.47$, $P = 0.06$].

In the three regions, the two isomers of sopromidine antagonized the increase in specific [^{35}S]GTPγ[S] binding induced by 1 μM imetit (Figure 5B). In the presence of 100 μM (highest concentration tested) of each of the two enantiomers, [^{35}S]GTPγ[S] binding had returned to control values (100%) in the cerebral cortex and striatum, and was even further decreased (by up to 15%) in the hypothalamus, thereby revealing the inverse agonist properties of the compounds (Figure 5B). Taking into account only the antagonistic part of the curves (i.e. above 100%) and an EC_{50} value of imetit of 2 ± 1 nM (not shown), the apparent K_i values found for the S enantiomer were roughly similar in the three regions (47 ± 14, 14 ± 0.6 and 23 ± 9 nM in the hypothalamus, cerebral cortex and striatum, respectively), whereas the apparent K_i values found for the R enantiomer increased from 21 ± 14 nM in the hypothalamus to 37 ± 35 nM in the cerebral cortex and 163 ± 92 nM in the striatum. The resulting S/R ratios increased from 2 in the hypothalamus, to 3 in the cerebral cortex and 8 in the striatum (Figure 5B). In agreement, two-way ANOVA revealed that the difference observed between the effects of the R and S enantiomers of sopromidine reached significance only in the striatum [$F(1,79) = 35.48$, $P < 0.0001$], *post hoc* analysis indicating a significant difference between the two enantiomers at 10 μM ($P < 0.01$) and 100 μM ($P < 0.001$) (Figure 5B).

Discussion

All the findings of this study support the assumption that the function of the autoreceptors modulating histaminergic neurons was not fulfilled by the long isoform of the H_3 receptor, but by a short form, such as the $H_{3(413)}$R isoform. These findings included: (i) the partial agonist effect of R-αMeHA on [^{3}H]AA release mediated by the long isoform has never been found either at native H_3 autoreceptors or at short isoforms; (ii) the stereoselectivity ratio of enantiomers of N^{α}Me-αClMeHA at autoreceptors was only fourfold lower at the functional $H_{3(413)}$ receptor isoform, but was much lower at the functional long isoform; (iii) this stereoselectivity of N^{α}Me-αClMeHA on [^{35}S]GTPγ[S] binding was higher in the hypothalamus, the region of origin of histaminergic neurons, than in the cerebral cortex or striatum; (iv) (R) sopromidine behaved as an antagonist with a similar potency at functional

Table 2
Compared properties of R(−) and S(+) sopromidine at the recombinant $rH_{3(445)}$ and $rH_{3(413)}$ receptor isoforms and at the rat H_3 autoreceptor

Condition	R(−) sopromidine $rH_{3(445)}$ receptor	$rH_{3(413)}$ receptor	H_3 autoreceptor[a]	S(+) sopromidine $rH_{3(445)}$ receptor	$rH_{3(413)}$ receptor	H_3 autoreceptor[a]
Alone	Full agonist (EC_{50} = 79 ± 34 nM)	Partial agonist (EC_{50} = 91 ± 78 nM)	nd	Inverse agonist (EC_{50} = 180 ± 90 nM)	Inverse agonist (EC_{50} = 340 ± 92 nM)	nd
With histamine	No effect (K_i > 100 μM)	Antagonist (K_i = 63 ± 40 nM)	Antagonist (K_i = 56 ± 22 nM)	Antagonist (K_i = 250 ± 120 nM)	Antagonist (K_i = 220 ± 100 nM)	Antagonist (K_i = 45 ± 11 nM)

[a]Values from Arrang *et al.* (1985a). The values at the two isoforms are derived from data shown in Figure 4.
nd, not determined.

$H_{3(413)}$ receptor isoforms and autoreceptors, but as a full agonist at the long isoform; (v) sopromidine isomers exhibited no stereoselectivity at autoreceptors and on [^{35}S]GTPγ[S] binding in the hypothalamus but showed some stereoselectivity in the cerebral cortex or striatum.

The observation that autoreceptors are the short receptor isoforms is consistent with their expression level in the brain. Functional (Schlicker *et al.*, 1994; Haas *et al.*, 2008), localization (Goodchild *et al.*, 1999; Anichtchik *et al.*, 2001; Pillot *et al.*, 2002) and lesion (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Anichtchik *et al.*, 2000) studies showed that the majority of H_3 receptors in the brain are not autoreceptors on histaminergic neurons, but are post-synaptic receptors or heteroreceptors present on other neuronal populations. In addition, in most brain areas from different species including human, the expression of the long isoform is largely predominant compared with shorter functional isoforms, with deletions in the third intracellular loop. such as the $H_{3(413)}$ receptor isoform (Tardivel-Lacombe *et al.*, 2000; Coge *et al.*, 2001; Drutel *et al.*, 2001; Morisset *et al.*, 2001; Rouleau *et al.*, 2004). Although the long isoform can be excluded, our study does not identify which short isoform(s) fulfill(s) the autoreceptor function. However, whereas all the other splice variants differ in composition between species (Hancock *et al.*, 2003), the expression of the $H_{3(413)}$ receptor isoform is the only one to be maintained in the brain from all species including rat (Morisset *et al.*, 2001), mouse (Rouleau *et al.*, 2004), guinea pig (Tardivel-Lacombe *et al.*, 2000), monkey (Strakhova *et al.*, 2008) and human (Coge *et al.*, 2001; Tardivel-Lacombe *et al.*, 2001). This may therefore indicate that this short isoform does play the role of autoreceptor including in human brains. The involvement of shorter variants such as the $H_{3(397)}$ receptor in rodent (Drutel *et al.*, 2001; Morisset *et al.*, 2001; Rouleau *et al.*, 2004), or the $H_{3(365)}$ receptor in human (Coge *et al.*, 2001; Wellendorph *et al.*, 2002; Bongers *et al.*, 2007), alone or with the $H_{3(413)}$ receptor isoform, cannot, however, be entirely ruled out. Unfortunately, the stereoselectivity of the human $H_{3(365)}$ receptor could not be evaluated in this study because no response was produced by this isoform in CHO cells (data not shown), as also reported in one of the earlier studies (Coge *et al.*, 2001).

The mechanisms leading a short, rather than the long, isoform to play the role of autoreceptor remain unknown. It is worth noting that our observations on the H_3 receptor resemble those on the D_2 dopamine receptor. The short isoform of the D_2 receptor also differs from the D_2 long isoform by a 29-amino acid deletion in the third cytoplasmic loop and also functions as an autoreceptor (Usiello *et al.*, 2000; Centonze *et al.*, 2002; Lindgren *et al.*, 2003). This functional selectivity was suggested on the basis of a predominant location of the short isoform in dopaminergic neurons (Khan *et al.*, 1998; Jomphe *et al.*, 2006) and/or from a differential coupling leading the short isoform to be preferred for the autoreceptor function (Senogles, 1994; Guiramand *et al.*, 1995; Liu *et al.*, 1996; Wolfe and Morris, 1999; Van *et al.*, 2007). These two suggestions may apply to the functional selectivity of H_3 receptors. However, whether histaminergic neurons selectively express the short isoforms of H_3 receptors is not yet known, inasmuch as transcripts of both long and short isoforms have been observed in the hypothalamus, their region of origin (Morisset *et al.*, 2001). Alternatively, the

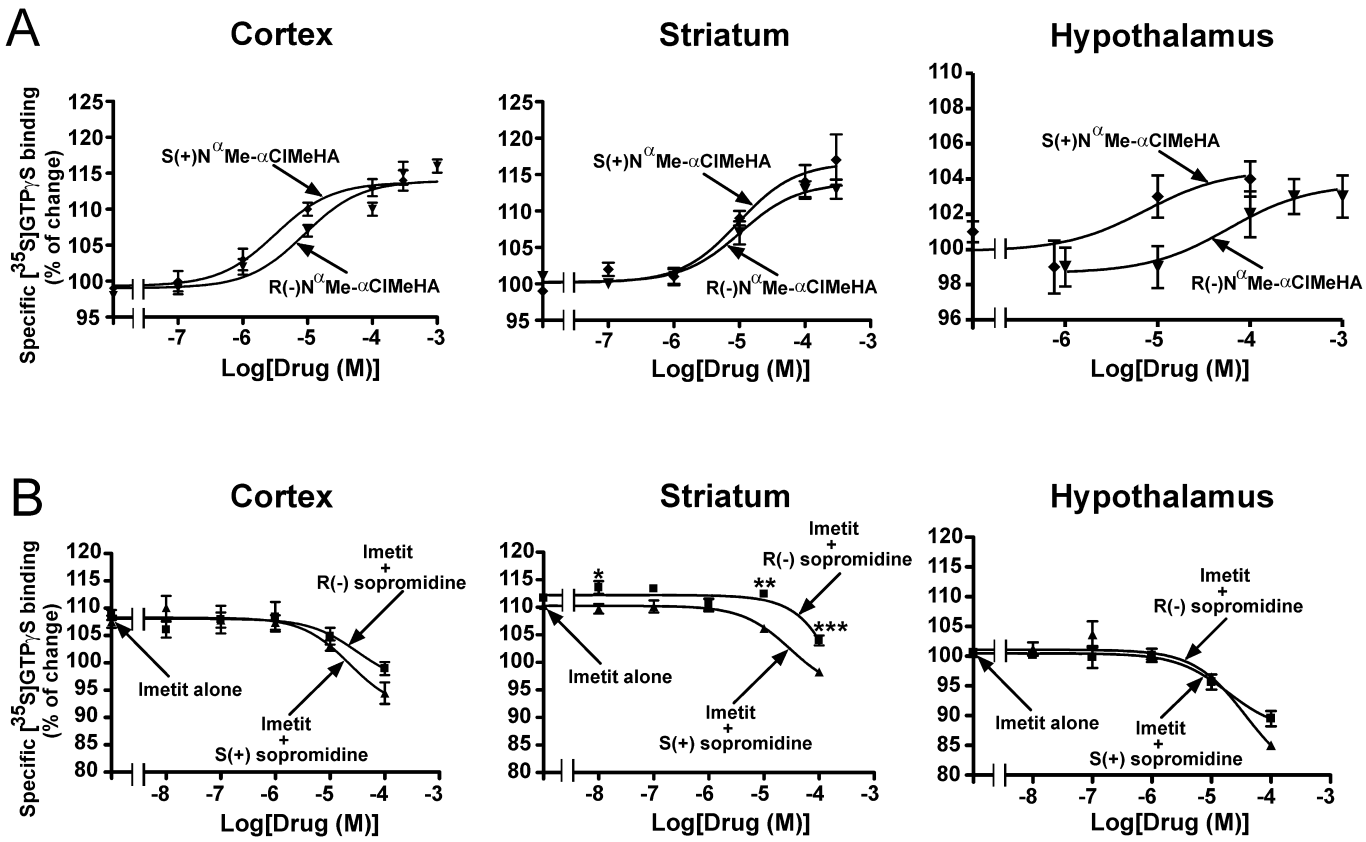

Figure 5

Effects of the R(–) and S(+) enantiomers of N$^{\alpha}$Me-αClMeHA and sopromidine on specific [^{35}S]GTPγ[S] binding to membranes from various rat brain regions. Membranes were incubated with 0.1 nM [^{35}S]GTPγ[S] in the presence of increasing concentrations of N$^{\alpha}$Me-αClMeHA enantiomers alone (A), or sopromidine enantiomers in the presence of 1 μM imetit (B). In order to prevent any interaction of the drugs with H_1 and H_2 receptors, all incubations were performed in the presence of 100 nM mepyramine and 10 μM cimetidine. Data are means ± SEM of 4–16 determinations from two to four separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from imetit + S(+) sopromidine.

autoreceptor function of short isoforms may result from their selective signalling properties. More interestingly, both H_3 (Takeshita *et al.*, 1998; Stevens *et al.*, 2001; Moreno-Delgado *et al.*, 2009) and D_2 (Wolfe and Morris, 1999) receptors inhibit high voltage-activated Ca^{2+} channels, which is likely to underlie their autoreceptor function on histamine and dopamine release respectively. However, the same coupling, that is, inhibition of the cAMP pathway, can be used by native H_3 autoreceptors to inhibit histamine synthesis in the brain (Moreno-Delgado *et al.*, 2009) and by both long and short isoforms. Therefore, whether H_3 and D_2 receptor short isoforms selectively couple to a common effector to inhibit histaminergic and dopaminergic neuron activity, respectively, remains to be shown.

This study confirms our studies on autoreceptors indicating that H_3 receptors prefer (+) enantiomers corresponding to the S-configuration of L-histidine, such as R-αMeHA or S-N$^{\alpha}$Me-αClMeHA (Arrang *et al.*, 1985a). However, we show for the first time that H_3 receptor isoforms differ markedly in their pharmacological profiles, indicating that they correspond to distinct H_3 receptor conformations. These different conformations resulting from differences in the third intracellular loop are not expected to generate significant differences in the affinity of the ligands, known to interact with the transmembrane domains. In agreement, differences between isoforms in binding studies were always limited (Coge *et al.*, 2001; Morisset *et al.*, 2001; Rouleau *et al.*, 2004). It is worth noting, however, that the affinity of betahistine was ~6-fold higher at the $rH_{3(413)}$ than at the $rH_{3(445)}$ receptor isoform, but very similar to its antagonist potency at rat autoreceptors (Arrang *et al.*, 1985b), which already led us to suggest that the function of autoreceptor was fulfilled by a short, rather than long, H_3 receptor isoform (Gbahou *et al.*, 2010). Moreover, it is well-established that H_3 receptors exist in multiple conformations displaying different pharmacological profiles (Gbahou *et al.*, 2003) and that H_3 receptor binding sites do not represent, at least solely, functional receptors. For example, inverse agonist radioligands label a much larger population of H_3 receptors than agonist radioligands (Witte *et al.*, 2006; Yao *et al.*, 2006; Mezzomo *et al.*, 2007), and agonist radioligands bind to both uncoupled and coupled states of the H_3 receptor (Arrang *et al.*, 1990). It explains that, whereas (R)- and (S)-αMeHA conserve their stereoselectivity in binding and functional studies (Arrang *et al.*, 1987; 1990), the stereoselectivity of the R and S enantiomers of N$^{\alpha}$Me-αClMeHA, observed here in functional

responses, was not observed in binding assays. All these considerations confirm that binding assays are not appropriate for the screening of compounds for a particular functional or therapeutic use.

Rather than having differences in affinity, H_3 receptor isoforms differ markedly at the level of their coupling to G proteins. Indeed, all of our data on the three studied responses can be explained by differences in agonist potency and/or intrinsic activity between the two isoforms. Firstly, R-αMeHA acted surprisingly as an intrinsic partial agonist at the long isoform. This effect was observed only for [^{3}H]AA release, a response with a low coupling efficiency of the H_3 receptor (Morisset *et al.*, 2000). For cAMP accumulation, where the H_3 receptor displays a higher coupling efficiency, a full agonist is expected to reach its maximal activity with only a partial occupancy of these receptors. A partial but potent agonist such as R-αMeHA is then expected to behave as an apparent full agonist by occupying more, if not all, functional receptors. In agreement, the relative potency of R-αMeHA was higher on [^{3}H]AA release than on cAMP accumulation.

Secondly, differences in coupling of isoforms also accounts for their strong differences in stereoselectivity observed with N$^\alpha$Me-αClMeHA enantiomers, both on cAMP formation mediated by the recombinant isoforms and on [^{35}S]GTPγ[S] binding to brain membranes. These differences were, in fact, generated by a lower potency of the non-preferred isomer, that is, the R(−), at the short isoform, whereas the potency of the preferred isomer, that is, the S(+), remained unchanged.

Thirdly, at both isoforms, the two enantiomers of sopromidine displayed opposite intrinsic properties, with the R(−) isomer behaving as agonist (full or partial) and the S(+) isomer acting as an inverse agonist. Their potency as agonist, antagonist or inverse agonist remained roughly similar, indicating that they stabilized conformations with similar binding properties, but different coupling properties. At autoreceptors inhibiting histamine release, we previously failed to detect both the partial agonist effect of R(−) sopromidine and the inverse agonist effect of S(+) sopromidine, and concluded that they were both behaving as full antagonists (Arrang *et al.*, 1985a), presumably because our system involved a lower density of receptors with no apparent constitutive activity (Morisset *et al.*, 2000). Therefore, in contrast to what we concluded from histamine release experiments, the H_3 receptor also displays a strong stereoselectivity for sopromidine enantiomers. Moreover, as found with N$^\alpha$Me-αClMeHA enantiomers, this stereoselectivity differed between the two isoforms, with the inverse agonist effect of S(+) sopromidine remaining unchanged, in contrast to the agonist effect of the R(−) isomer, which was full at the long isoform but very partial at the short isoform. [^{35}S]GTPγ[S] binding showed that this difference in stereoselectivity of isoforms also occurred in the brain. The absence of stereoselectivity of sopromidine tested as antagonist against imetit in the hypothalamus is consistent with the high density of autoreceptors in this region, both isomers being expected to act as apparent antagonists with similar potencies. In the cerebral cortex and striatum, the presumably higher density of long isoforms is likely to account for the stereoselectivity observed in these two regions, the agonist property of the R(−) isomer at these long isoforms counteracting its apparent antagonist properties at short isoforms. In agreement, this stereoselectivity was mainly generated by a decrease in the potency of the R(−) isomer in the cerebral cortex and striatum compared with the hypothalamus, whereas the potency of the S(+) isomer remained roughly the same in all three regions.

It is worth noting that these differences in coupling of H_3 receptor isoforms may be ligand-dependent because, in contrast to the compounds used in the present study, the two enantiomers of αMeHA revealed a similar stereoselectivity of rodent and human H_3 receptor isoforms (Arrang *et al.*, 1987; 1990; Wulff *et al.*, 2002; Hancock *et al.*, 2003).

In conclusion, the present findings show that short, but not long, isoforms fulfil the function of autoreceptor and thereby confirm the hypothesis that H_3 receptor isoforms have distinct functional roles in the brain. The roles played by the long isoform remain to be explored, but its transcripts, being predominant in most brain regions, may encode for the numerous receptors present on neurons other than histaminergic neurons, either at the post-synaptic level (somatodendritic receptors) or pre-synaptic level (heteroreceptors) (Pillot *et al.*, 2002). The pharmacological differences that we report here between isoforms indicate that it should be possible to identify ligands selective for each of them. Such ligands should then be helpful in discriminating between the selective functions of isoforms at the pre- and post-synaptic levels. Numerous pharmaceutical companies have invested considerable efforts in the clinical development of inverse agonists at the H_3 receptor, as possible treatments for wakefulness and cognition disorders (narcolepsy, Alzheimer's disease and attention deficit hyperactivity disorder) (Hancock and Fox, 2004; Passani *et al.*, 2004; Celanire *et al.*, 2005; Leurs *et al.*, 2005; Esbenshade *et al.*, 2006; Arrang *et al.*, 2007; Parmentier *et al.*, 2007; Lin *et al.*, 2008), but these drugs are not selective for any isoform. The clinical development of compounds that selectively target autoreceptors may enhance their therapeutic efficacy, in the above-mentioned disorders, as well as in other more controversial indications including food intake disorders (Hancock, 2003), seizures (Kamei, 2001) or schizophrenia (Southam *et al.*, 2009; Burban *et al.*, 2010; Motawaj and Arrang, 2011). Moreover, because no compound has yet been successfully developed into clinical use, the possible side effects of H_3 receptor inverse agonists remain unknown but may be decreased with compounds selective for short isoforms.

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Conflict of interest

None.

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