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RESEARCH PAPER

A single-point mutation (Ala280Val) in the third intracellular loop alters the signalling properties of the human histamine H_3 receptor stably expressed in CHO-K1 cells

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BACKGROUND AND PURPOSE

An alanine to valine exchange at amino acid position 280 (A280V) in the third intracellular loop of the human histamine H_3 receptor was first identified in a patient suffering from Shy–Drager syndrome and later reported as a risk factor for migraine. Here, we have compared the pharmacological and signalling properties of wild-type (hH_3R_{WT}) and A280V mutant (hH_3R_{A280V}) receptors stably expressed in CHO-K1 cells.

EXPERIMENTAL APPROACH

The hH_3R_{A280V} cDNA was created by overlapping extension PCR amplification. Receptor expression and affinity were assessed by radioligand (N-α-[methyl-^{3}H]-histamine) binding to cell membranes, and receptor function by the inhibition of forskolin-induced cAMP accumulation and stimulation of ERK1/2 phosphorylation in intact cells, as well as stimulation of [^{35}S]-GTPγS binding to cell membranes.

KEY RESULTS

Both receptors were expressed at similar levels with no significant differences in their affinities for H_3 receptor ligands. Upon activation the hH_3R_{WT} was significantly more efficacious to inhibit forskolin-induced cAMP accumulation and to stimulate [^{35}S]-GTPγS binding, with no difference in pEC_{50} estimates. The hH_3R_{WT} was also more efficacious to stimulate ERK1/2 phosphorylation, but this difference was not significant. The inverse agonist ciproxifan was more efficacious at hH_3R_{WT} to reduce [^{35}S]-GTPγS binding but, for both receptors, failed to enhance forskolin-induced cAMP accumulation.

CONCLUSIONS AND IMPLICATIONS

The A280V mutation reduces the signalling efficacy of the human H_3 receptor. This effect may be relevant to the pathophysiology of disorders associated with the mutation.

LINKED ARTICLES

This article is part of a themed issue on Histamine Pharmacology Update. To view the other articles in this issue visit http://dx.doi.org/10.1111/bph.2013.170.issue-1

Abbreviations
NHMA, N-α-methyl histamine; RAMH, (*R*)-α-methyl histamine

Introduction

In the mammalian brain histamine regulates several functions by acting at four different GPCRs, H_1–H_4 (Haas *et al.*, 2008; receptor nomenclature follows Alexander *et al.,* 2011). Histamine H_3 receptors are primarily expressed on nerve terminals, where they regulate the synthesis and release of histamine as well as the release of other neuroactive substances namely ACh, dopamine, noradrenaline, 5-HT, glutamate, GABA and substance P (Feuerstein, 2008). There is also evidence for post-synaptic H_3 receptors in the striatum, cerebral cortex and hippocampus (Pillot *et al.*, 2002).

Histamine H_3 receptors couple to $G\alpha_{i/o}$ proteins and thus trigger several signalling pathways that include inhibition of adenylyl cyclase, inhibition of voltage-operated Ca^{2+} channels, activation of phospholipase A_2 (PLA_2), modulation of the MAPK pathway and activation of the Akt/glycogen synthase kinase-3β axis (Bongers *et al.*, 2007)

The originally cloned human H_3 (hH_3) receptors cDNA encoded a 445 amino acid protein (Lovenberg *et al.*, 1999) and in 2002, Wiedemann *et al.* reported a variation in the genomic sequence of the H_3 receptors of a patient suffering from Shy–Drager syndrome, currently classified as multiple system atrophy with orthostatic hypotension (Jecmenica-Lukic *et al.*, 2012). The identified variation in the hH_3 receptors was a cytosine to thymine transition at nucleotide position 839 with respect to the start codon, which results in an alanine to valine exchange at amino acid position 280 in the third intracellular loop (Wiedemann *et al.*, 2002; see Supporting Information Figure S1). Further, a recent report by Millán-Guerrero *et al.* (2011) indicated that in a Mexican population (147 cases and 186 controls) the A280V mutation was associated with increased risk of migraine.

In this work we therefore set out to compare the pharmacological and signalling properties of wild-type (hH_3R_{WT}) and A280V mutant (hH_3R_{A280V}) receptors by stably expressing them in CHO-K1 cells. A preliminary account of this work has been presented in abstract form to the European Histamine Research Society (Flores-Clemente *et al.*, 2012).

Methods

Generation of the A280V mutant hH_3 receptor

The hH_3R_{A280V} cDNA was created by overlapping extension PCR amplification essentially as described by Horton *et al.* (1993). Amplifications were carried out in 25 μL incubations containing 0.8 mM dNTPs, 0.4 μM each primer, 0.5 U *Pfu* DNA polymerase (Fermentas, Glen Burnie, MD, USA) and either 100 ng of hH_3R_{WT} for initial amplifications or gel purified fragments from the amplifications as templates. The mutated DNA fragment was reintroduced into hH_3R_{WT} backbone after digestion of unique restriction sites BstEII (position 405 relative to start codon) and NotI (vector polylinker). The sequence of the amplified fragment was verified both by restriction analysis and automated sequencing performed at FESI-UNAM., Mexico.

Cell culture and transfection

CHO-K1 cells were cultured in DMEM Nutrient F-12 (DMEM-F12) mix supplemented with 10% FBS and 1% antibiotic/antimycotic solution (Gibco Life Technologies, Grand Island, NY, USA). For stable transfection, CHO-K1 cells (70–80% confluence) were transfected with cDNA constructs using Lipofectamine 2000 (Invitrogen Life Technologies, Grand Island, NY, USA), selected in 600 $\mu g \cdot mL^{-1}$ geneticin (G-418), tested for receptor expression by radioligand binding and maintained in the presence of 400 $\mu g \cdot mL^{-1}$ geneticin.

N-α-(methyl-^{3}H)-histamine [(^{3}H)-NMHA] binding assays

Cells grown in plastic Petri dishes (100 mm diameter) were scrapped and homogenized in ice-cold hypotonic buffer (10 mM Tris-HCl, 1 mM EGTA, pH 7.4) and lysates centrifuged (42,000× *g*, 20 min at 4°C). Pellets were re-suspended in incubation buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) and binding determinations (20 μg protein aliquots) were carried out and analysed as described in detail elsewhere (Osorio-Espinoza *et al.*, 2011). Protein contents were determined by the bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA), using BSA as standard.

Measurement of cAMP accumulation in intact cells

Cells, grown in 24-well plates, were incubated (37°C) in 250 μL Krebs–Ringer–HEPES (KRH) buffer (composition (mM): NaCl 113, $NaHCO_3$ 25, KCl 3, $MgCl_2$ 1, KH_2PO_4 1, $CaCl_2$ 1.8, D-glucose 11, HEPES 20; pH 7.4 with NaOH) containing 1 mM IBMX. After 15 min, forskolin was added in a 10 μL volume and incubations continued for a further 15 min. Final forskolin concentrations were 10 or 3 μM for the test of agonists or inverse agonists, respectively, and where required H_3 receptor agonists were added 5 min before forskolin.

Incubations were terminated with 25 μL ice-cold 1 M HCl. After neutralization with 25 μL 1 M NaOH and 100 μL 1 M Tris-HCl (pH 7.4), endogenous cAMP was determined by a competition assay for which 50 μL samples were incubated in 125 μL of incubation buffer (composition (mM): 50 Tris-HCl, 100 NaCl, 5 EDTA, 5 $mg \cdot mL^{-1}$ BSA, pH 7.0 at 4°C) containing the PKA regulatory subunit (0.5 UI per sample) and [^{3}H]cAMP (10 nM). After 2.5 h at 4°C, incubations were terminated by filtration over GF/B filters pre-soaked in 0.3% polyethylenimine followed by three subsequent washes with 1 mL ice-cold deionized water. Retained radioactivity was determined by liquid scintillation and the amount of endogenous cAMP present in each sample was calculated by using a standard cAMP curve (10^{-12}–10^{-5} M).

[^{35}S]-GTPγS binding assay

Assays were carried out essentially as described by Gardner *et al.* (1996). Briefly, cells were scraped and lysed in ice-cold hypotonic buffer (20 mM HEPES, 2 mM EDTA, pH 7.4) containing a protease inhibitor cocktail (1:100 v : v; Sigma Aldrich, Mexico City, Mexico), followed by centrifugation at 42,000× *g* (20 min, 4°C). The pellet (membranes) was

re-suspended in 10 mL HEPES buffer (20 mM HEPES, 100 mM NaCl, pH 7.4) containing adenosine deaminase (10 UI·mL^{-1}) and incubated for 30 min at 30°C. Ice-cold HEPES buffer (20 mL) was added and the membrane suspension was centrifuged as described earlier. The resulting pellet was re-suspended and incubated (20 µg protein aliquots) in the absence and presence of drugs under test in 500 µL HEPES buffer containing 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 10 µM GDP, 1 mg·mL^{-1} BSA, 0.2 mM EDTA, 10 mg·mL^{-1} saponin and 50 pM [^{35}S]-GTPγS. Non-specific binding was determined in the presence of 10 µM unlabelled GTPγS. After 30 min at 30°C, the reaction was terminated by rapid filtration through GF/B filters with four washes of 1 mL ice-cold HEPES buffer. Radioactivity bound to the filters was quantified by liquid scintillation counting.

Detection of H_3 receptor-induced phosphorylation of ERK1/2 (p44/p42-ERK) by Western blotting

Cells, incubated overnight in DMEM-F12 medium containing 0.1% FBS, were washed with KRH buffer and incubated at 37°C with the H_3 receptor agonist (*R*)-α-methylhistamine (RAMH). After rinsing twice with pre-warmed KRH solution and once with ice-cold solution, cells were scraped into lysis buffer [20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1% Triton X-100, 10% deoxycholate, 10% SDS, 1% glycerol, 150 mM NaCl and 1% (v : v) protease inhibitor cocktail]. The samples were cleared by centrifugation and samples (30 µg protein) were resolved by SDS-PAGE (10% gel) and then transferred onto a PVDF membrane.

Blots were blocked (1 h at 22°C) with Tris-buffered saline containing Tween-20 (0.1% v : v; T-BST) and 5% non-fat dry milk, before incubation overnight at 4°C with primary antibodies (rabbit anti phospho-ERK1/2 or anti total-ERK1/2, Cell Signaling, Danvers, MA, USA; 1:1000 dilution in T-BST containing 1.5% BSA). After rinsing with T-BST, membranes were incubated for 1 h at 22°C with a secondary antibody (goat anti-rabbit IgG coupled to HRP; 1:5000 in T-BST/1.5% BSA). Membranes were rinsed with T-BST, developed with chemiluminescence (Enhanced ChemiLuminescent Substrate; Biorad, Hercules, CA, USA) and quantified by densitometry analysis with the Kodak Image Station 4000R (Eastman Kodak, Rochester, NY, USA) and ImageJ software (National Institutes of Health, USA). Target bands were expressed quantitatively by normalization to the intensity of the non-phosphorylated ERK1/2 signal on the same lane. Protein loading was also controlled by actin immunodetection.

Data analysis

Results are presented as the means ± s.e.m. of the indicated number of experiments. Statistical comparisons were performed with Student's unpaired *t*-test or one-way ANOVA followed by Dunnett's test, as appropriate (Prism 5.0 software; GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

Materials

The following drugs and reagents were purchased from Sigma Aldrich: A-331440 (4'-[3-[(3(R)-dimethylamino-1-pyrrolidinyl]propoxy]-[1,1-biphenyl]-4'-carbonitrile dihydrochloride), adenosine deaminase (from bovine spleen), cAMP, ciproxifan hydrochloride, clobenpropit dihydrobromide, DTT, GDP, IBMX, geneticin, GTPγS, histamine dihydrochloride, PKA regulatory subunit (from bovine heart), (*R*)-α-methylhistamine dihydrochloride and saponin. N-α-[methyl-^{3}H]-histamine (85.4 Ci·mmol^{-1}), [^{35}S]GTPγS (1250 Ci·mmol^{-1}) and [^{3}H]-cAMP ([2,8-^{3}H]-adenosine 3',5'-cyclic phosphate, 34 Ci·mmol^{-1}) were from Perkin Elmer (Boston, MA, USA).

Results

Binding characteristics of hH_3 receptors stably expressed in CHO-K1 cells

Specific [^{3}H]-NMHA binding to cell membranes, yielded maximum binding (B_{max}) 203 ± 34 and 176 ± 26 fmol·mg·protein^{-1} (mean ± SEM; four experiments, Figure 1A) for hH_3R_{WT} and hH_3R_{A280V}, respectively, with no statistical difference ($P = 0.25$, Student's *t*-test). Estimates for the equilibrium dissociation constant (K_d) were 0.86 ± 0.23 and 0.83 ± 0.24 nM respectively ($P = 0.46$).

For both receptors, a set of agonists (histamine, imetit, immepip and RAMH) and antagonists/inverse agonists (A-331440, ciproxifan, clobenpropit and thioperamide) inhibited [^{3}H]-NMHA binding in a concentration-dependent manner (Figure 1B,C). Estimates for the inhibition constants (-log *K*i, p*K*i; Cheng and Prusoff, 1973) are shown in Table 1. There was no significant difference for any of the ligands tested between the native (wild type) and mutant receptors. Further, the affinities of both receptors were in good accord with values previously reported for the expression of cloned hH_3 receptors in CHO-K1 cells (Ligneau *et al.*, 2000; Cogé *et al.*, 2001; Table 1).

Agonist-induced inhibition of forskolin-induced cAMP accumulation

Forskolin-stimulated cAMP accumulation in a similar manner for CHO-K1-hH_3R_{WT} and CHO-K1-hH_3R_{A280V} cells (Supporting Information Figure S2), and the effect of 10 µM of forskolin was significantly reduced by the H_3 receptor agonist RAMH (Figure 2A). However, maximum inhibition was significantly lower in cells expressing the mutant receptor (Table 2). The estimates for half-maximal effective concentrations (pEC_{50}) showed a tendency to a lower potency for CHO-K1-hH_3R_{A280V} cells, but the difference was not statistically significant, as shown in Table 2.

Agonist-induced [^{35}S]-GTPγS binding

The ability of GPCRs to activate G-proteins is also studied by determining [^{35}S]-GTPγS binding to cell membranes. Maximum stimulation evoked by the agonist RAMH was significantly higher in membranes from CHO-K1-hH_3R_{WT} cells (Figure 2B and Table 2, and Supporting Information Figure S3A), with no significant difference in the pEC_{50} estimates.

Agonist-induced ERK1/2 phosphorylation

Activation of transfected H_3 receptors leads to ERK1/2 phosphorylation (Bongers *et al.*, 2007), and in both CHO-K1-hH_3R_{WT} and CHOK1-hH_3R_{A280V} cells, incubation (1–15 min)

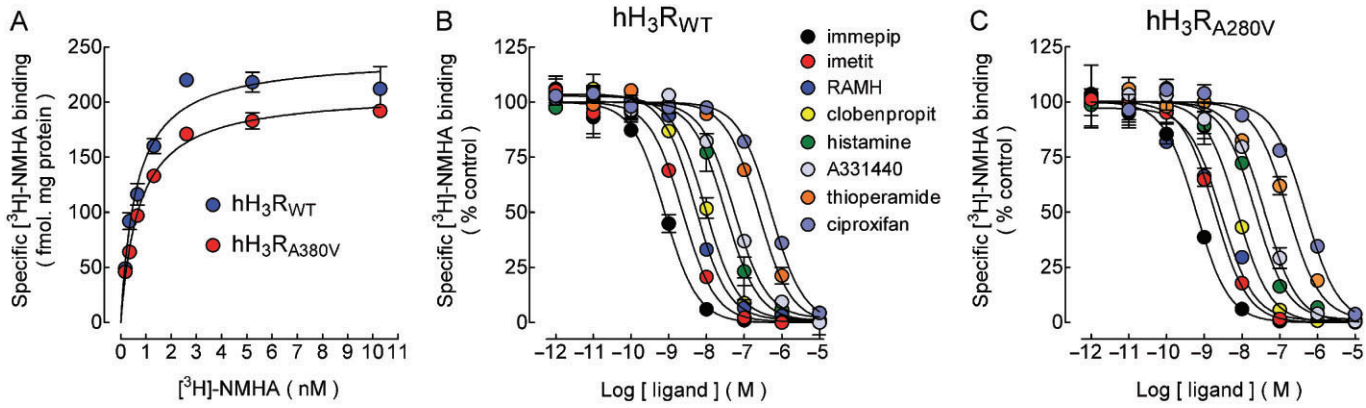

Figure 1

Binding of *N*-α-[methyl-^{3}H]histamine ([^{3}H]-NMHA) to membranes from CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells. (A) Saturation binding. Cell membranes were incubated with the indicated concentrations of [^{3}H]-NMHA and specific receptor binding was determined by subtracting the binding in the presence of 10 μM histamine from total binding. Points are means ± SEM from triplicate determinations from a single experiment, which was repeated a further three times. The line drawn is the best fit to a hyperbola. Best-fit values for the equilibrium dissociation constant (K_d) and maximum binding (B_{max}) are given in the text. (B,C) Inhibition by H_3 receptor ligands. Membranes from CHO-K1-hH_3R_{WT} (B) or CHO-K1-hH_3R_{A280V} cells (C) were incubated with 1.5 nM [^{3}H]-NMHA and the indicated drug concentrations. Values are expressed as percentage of control specific binding and are averages ± ranges of duplicates from a single experiment, repeated a further two to four times with similar results. The line drawn is the best fit to a logistic equation for a one-site model. pK_i values calculated from the best-fit IC_{50} estimates are compared in Table 1.

Table 1

Affinities of hH_3R_{WT} or hH_3R_{A280V} for selective ligands

	pK$_i$ values			
	This study		Ligneau *et al.*, 2000	Cogé *et al.*, 2001
	hH_3R_{WT}	hH_3R_{A280V}		
Agonists				
Histamine	8.30 ± 0.10	8.38 ± 0.12 (P = 0.623, n = 4)	7.89	8.24 ± 0.01
Immepip	9.51 ± 0.03	9.58 ± 0.01 (P = 0.091, n = 3)	ND	9.60 ± 0.15
Imetit	9.23 ± 0.19	9.29 ± 0.05 (P = 0.775, n = 3)	ND	9.37 ± 0.22
RAMH	8.92 ± 0.11	9.05 ± 0.10 (P = 0.431, n = 3)	8.56	8.73 ± 0.09
Antagonists/inverse agonists				
A-331440	7.75 ± 0.04	7.77 ± 0.08 (P = 0.834, n = 3)	ND	ND
Ciproxifan	7.17 ± 0.04	7.21 ± 0.10 (P = 0.729, n = 3)	7.33	7.26 ± 0.02
Clobenpropit	8.79 ± 0.08	8.64 ± 0.12 (P = 0.329, n = 5)	8.62	9.27 ± 0.05
Thioperamide	7.34 ± 0.13	7.37 ± 0.05 (P = 0.840, n = 3)	7.22	7.70 ± 0.07

Values are pK_i estimates (-Log_{10} of the inhibition constant, K_i) and correspond to means ± SEM from the indicated number of determinations. Figures in parentheses are P values (Student's t-test) for the comparison between hH_3R_{WT} and hH_3R_{A280V} cells. Determinations reported in Ligneau *et al.* (2000) and Cogé *et al.* (2001) were performed with the hH_3 receptor stably expressed in CHO-K1 cells. Values from Ligneau *et al.* (2000) were transformed to pK_i estimates from the K_i values provided. ND, not determined.

with the agonist RAMH resulted in increased ERK1/2 phosphorylation over basal levels that reached a peak between 2.5 and 5 min to decay steadily afterwards (Supporting Information Figure S4A). At 5 min incubations, the effect was concentration-dependent (Supporting Information Figure S4B), with a tendency for higher efficacy in CHOK1-hH_3R_{WT} cells, although the effect was not significant (Table 2).

Effect of inverse agonists on [^{35}S]-GTPγS binding and forskolin-induced cAMP accumulation

Native (wild type) and cloned H_3 receptors may exhibit spontaneous or constitutive activity (Arrang *et al.*, 2007), and for hH_3 receptors expressed in CHO cells such an activity is apparent in cells expressing 250–400 fmol mg^{-1} protein of the

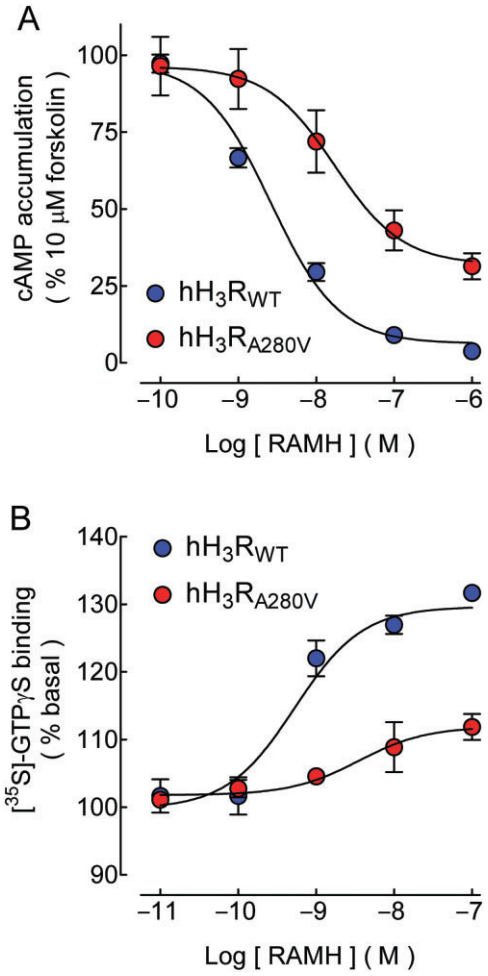

Figure 2

Effect of H_3 receptor activation on forskolin-induced cAMP accumulation and [^{35}S]-GTPγS binding. (A) Forskolin-induced cAMP accumulation. CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells were pre-incubated (15 min) with 1 mM IBMX and then exposed for 15 min to forskolin (10 µM). Where required the H_3 receptor agonist RAMH was added 5 min before forskolin. Values are expressed as percentage of the response to forskolin, after basal subtraction, and correspond to the means ± SEM from four replicates from a representative experiment, repeated a further four times with similar results. The line drawn is the best fit to a logistic equation. Estimates for maximum effect (E_{max}) and pEC_{50} are compared in Table 2. Raw data from a representative experiment are presented in Supporting Information Figure S2. (B). [^{35}S]-GTPγS binding. Membranes from CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells were incubated (30 min) with 50 pM [^{35}S]-GTPγS in the presence and absence of the indicated concentrations of RAMH. Values are expressed as percentage of basal [^{35}S]-GTPγS binding after subtraction of non-specific binding and correspond to the means ± SEM from three replicates from a representative experiment, repeated a further seven times with similar results. The line drawn is the best fit to a logistic equation. Estimates for maximum stimulation and pEC_{50} are compared in Table 2. Basal [^{35}S]-GTPγS binding was 4993 ± 226 and 5179 ± 43 dpm for membranes from CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells, respectively. Raw data from a representative experiment are presented in Supporting Information Figure S3.

hH_3 receptor (Rouleau *et al.*, 2002). Because the reduced efficacy of hH_3R_{A280V} observed in our experiments may rely on higher intrinsic activity, the effect of antagonists/inverse agonists was tested in functional assays.

For both native and mutant receptors, the H_3 receptor antagonist/inverse agonist ciproxifan (Leurs *et al.*, 2005) reduced [^{35}S]-GTPγS binding to cell membranes. The effect was modest (Figures 3A and Supporting Information Figure S3B), but significantly higher for membranes from CHO-K1-hH_3R_{WT} cells (–12.03 ± 0.29 vs. –5.85 ± 0.60% of basal for CHOK1-hH_3R_{A280V} membranes; $P < 0.001$, $n = 4$, Student's *t*-test), without a significant effect in agonist potency (pEC_{50} values 9.06 ± 0.45 and 9.09 ± 0.73, respectively; $P = 0.964$). The errors associated to pEC_{50} values are large, most likely due to the small window for the ciproxifan effect which hampers the estimate accuracy.

Forskolin-stimulated cAMP accumulation increased in a concentration-dependent manner (Supporting Information Figure S2), but the slope was significantly higher between 1 and 10 µM than between 10 and 30 µM (2.05 ± 0.21 and 0.53 ± 0.13, respectively; $P < 0.001$, Student's *t*-test). For testing receptor constitutive activity, the forskolin concentration was therefore set at 3 µM in order to reliably assess the possible enhancing action of inverse agonists.

In contrast to [^{35}S]-GTPγS binding to cell membranes, for both CHO-K1-hH_3R_{WT} and CHOK1-hH_3R_{A280V} cells ciproxifan had no significant effect on forskolin-induced cAMP accumulation over a wide range of concentrations (10^{-11}–10^{-6} M, three experiments; data not shown). Other H_3 receptor inverse agonists (clobenpropit, thioperamide and A-331440) also failed to modify cAMP accumulation at concentrations of 0.1 and 1 µM, as shown in Figure 3C (panels B and C).

Discussion and conclusions

The main finding of this work is that the A280V mutation on the hH_3 receptor modifies its signalling efficacy without affecting the binding characteristics.

Effect of the A280V mutation on H_3 receptor binding and functional characteristics

Binding determinations indicated that the A280V mutation did not affect hH_3 receptor expression or its affinity for a series of selective ligands. The latter result was not unexpected because the mutation is not located on any of the receptor regions primarily involved in ligand binding, namely trans-membrane domains 3, 5 and 6 (Uveges *et al.*, 2002; Ishikawa *et al.*, 2010; Kim *et al.*, 2011). In contrast, the mutant receptor was less efficacious to inhibit forskolin-stimulated cAMP accumulation and to stimulate [^{35}S]-GTPγS binding, indicating that the mutation did affect the signalling properties of the receptor.

There was also a trend for lower efficacy for the mutant receptor for the stimulation of ERK1/2 phosphorylation, but the effect did not reach statistical significance. Receptor-induced ERK1/2 phosphorylation can be due either to Gβγ subunits or β-arrestins bound to activated receptors acting as a signalling scaffold (Gutkind, 2000; DeWire *et al.*, 2007), but the fast response induced by hH_3R_{WT} and hH_3R_{A280V} indicates

Table 2

Effect of receptor activation on forskolin-induced cAMP accumulation and [^{35}S]-GTPγS binding

	hH_3R_{WT}	hH_3R_{A280V}	*P*
cAMP accumulation			
E_{max} (%)	−93.9 ± 2.6	−57.2 ± 8.4	0.003
pEC_{50}	8.22 ± 0.35	7.75 ± 0.05	0.224
[^{35}S]-GTPγS binding			
E_{max} (% basal)	129.2 ± 2.6	112.6 ± 2.7	<0.001
pEC_{50}	8.50 ± 0.14	9.00 ± 0.32	0.174
ERK1/2 phosphorylation			
E_{max} (% basal)	302 ± 63	217 ± 31	0.155
pEC_{50}	7.94 ± 0.17	7.49 ± 0.19	0.128

Values are means ± SEM from five (cAMP accumulation), eight ([^{35}S]-GTPγS binding) or four (ERK1/2 phosphorylation) experiments. Statistical comparisons were carried out with Student's *t*-test.

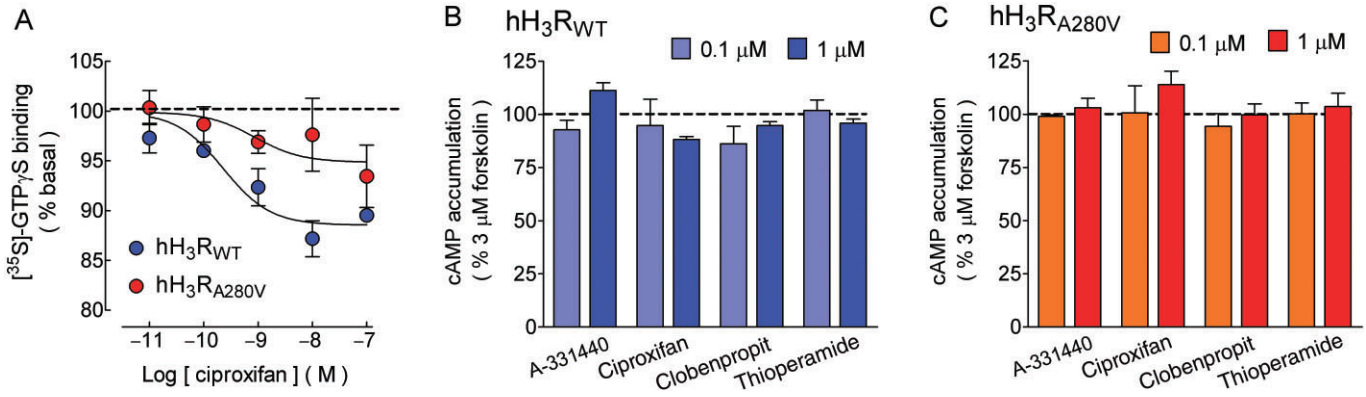

Figure 3

Effect of the H_3 receptor inverse agonists on [^{35}S]-GTPγS binding and forskolin-induced cAMP accumulation. (A) [^{35}S]-GTPγS binding. Membranes from CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells were incubated (30 min) with 50 pM [^{35}S]-GTPγS in the presence and absence of the indicated concentrations of ciproxifan. Values are expressed as percentage of basal [^{35}S]-GTPγS binding after subtraction of non-specific binding and correspond to the means ± SEM from three replicates from a representative experiment, repeated a further three times with similar results. The line drawn is the best fit to a logistic equation. Best-fit values for maximum effect (E_{max}) and pEC_{50} are given in the text. Basal [^{35}S]-GTPγS binding was 3527 ± 54 and 4487 ± 77 dpm for membranes from CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells respectively. Raw data from a representative experiment are presented in Supporting Information Figure S3. (B,C) Forskolin-induced cAMP accumulation. CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells were pre-incubated (15 min) with 1 mM IBMX and then exposed for 15 min to forskolin (3 μM). Where required, H_3 receptor inverse agonists (0.1 and 1 μM) were added 5 min before forskolin. Values are expressed as percentage of the response to forskolin, after basal subtraction, and correspond to the means ± SEM from three or four experiments for 0.1 or 1 μM of the inverse agonists, respectively. None of the values in the presence of H_3 receptor inverse agonists was significantly different from control values ($P > 0.05$, one-way ANOVA and *post hoc* Dunnett's test).

that the effect is more likely to rely on βγ complexes than on β-arrestin recruitment because the latter is a delayed response with a peak at 30 min incubations (Rosethorne and Charlton, 2011). The lack of difference in the efficacy of hH_3R_{WT} and hH_3R_{A280V} for the stimulation of ERK1/2 phosphorylation may reflect the distinct sensitivity of the pathways to the signalling molecules activated upon receptor stimulation.

Native and cloned H_3 receptors show constitutive activity (Arrang *et al.*, 2007), and in CHO cells spontaneous activity of the hH_3 receptor, evaluated by ciproxifan-induced inhibition of basal [^{35}S]-GTPγS binding is apparent from a density of 250 fmol receptor per mg protein (Rouleau *et al.*, 2002). Our [^{35}S]-GTPγS binding data indicate that both receptors do have constitutive activity when expressed in CHO-K1 cells, although to a modest extent. However, H_3 receptor inverse agonists failed to enhance forskolin-induced cAMP accumulation. This discrepancy may rely on the activated G proteins detected by [^{35}S]-GTPγS binding not yielding the concentration required for a significant inhibitory effect upon adenylyl cyclases stimulated by forskolin.

There is no direct evidence from this study as to the molecular mechanism responsible for the effect of the A280V

mutation on H_3 receptor function, although the change in agonist efficacy but not in potency suggests that the mutation is hampering the receptor ability to activate G-proteins. The A280V mutation is located in the receptor third intracellular loop (Figure S1) which plays a key role in determining receptor/G-protein coupling (Wess, 1998). Activated receptors catalyse GDP release from G-protein heterotrimers and this is the rate-limiting step in G-protein activation and, consequently, the activation of downstream signalling proteins (Oldham and Hamm, 2007). Because both alanine and valine are neutral amino acids, the A280V mutation confers no change in charge, but in size (71.1 and 99.1 daltons, and 0.067 and 0.105 nm^3, respectively; Zamyatnin, 1972), and one possible explanation for the reduced efficacy of the mutant receptor is a steric reduction in the access of the C-terminus of the $G\alpha_{i/o}$ protein to sites in the cytoplasmic face of the receptor trans-membrane helices required for G-protein stimulation (Oldham and Hamm, 2007).

Possible implications of the A280V mutation for the pathophysiology of the Shy–Drager syndrome and migraine

This study originated from reports that the A280V mutation may be involved in the pathophysiology of two neurological disorders, the Shy–Drager syndrome (Wiedemann *et al.*, 2002) and migraine (Millán-Guerrero *et al.*, 2011).

The Shy–Drager syndrome is now recognized as a form of multiple system atrophy, a sporadic and progressive neurological disease characterized by autonomic dysfunction, Parkinsonism and ataxia in any combination (Jecmenica-Lukic *et al.*, 2012). In the patient in whom the A280V mutation was first identified, a reduction in both noradrenaline plasma level and urinary excretion of the amine and its metabolites was found, and a link between the H_3 receptor mutation and altered noradrenaline release was suggested (Wiedemann *et al.*, 2002). Because H_3 receptor activation reduces depolarization-evoked noradrenaline release from sympathetic nerve terminals (Silver *et al.*, 2002), the decrease in noradrenaline plasma levels cannot be directly explained by the reduced efficacy of the mutant receptor reported herein, which would instead lead to increased noradrenaline release from peripheral neurones, in analogy with the enhanced release from sympathetic nerve endings observed in mice lacking H_3 receptors (Koyama *et al.*, 2003). Further, orthostatic hypotension has been mainly related to dysfunction of the catecholaminergic rostral ventrolateral medulla nuclei (Colosimo, 2011), and one possibility to explain the alterations in arterial pressure is that the reduced signalling efficacy of mutant H_3 auto-receptors enhances histamine release resulting in augmented activation of H_3 hetero-receptors located on central noradrenergic/adrenergic neurones (Pillot *et al.*, 2002) and thus in inhibition of neuronal firing. Alternatively, reduced receptor efficacy might lead to diminished H_3 receptor-mediated inhibition of neurotransmitter release and increased auto-receptor activation in cathecolaminergic neurones resulting in decreased neuronal activity. In turn, reduced activity of the rostral ventrolateral medulla nuclei would result in decreased sympathetic vasomotor tone (Guyenet, 2006).

Migraine is a neurovascular disorder involving activation of the trigemino-vascular system with the primary dysfunction located in brainstem centres regulating vascular tone and pain sensation (Cutrer, 2010). Its frequency is significantly higher in patients with allergic (histamine-driven) disease (Ku *et al.*, 2006; Aamodt *et al.*, 2007) and plasma histamine levels are significantly elevated in migraine patients (Heatley *et al.*, 1982). Recently, Millán-Guerrero *et al.* (2011) reported that the A280V mutation in the hH_3 receptor was significantly associated with increased risk of migraine with V allele frequencies of 6.46% and 2.68% for cases and controls, respectively (odds ratio 2.67), and frequencies of the V/V and V/A genotypes 12.92% and 3.22% in migraine patients and controls (odds ratio 4.45). This group also reported that S.C. injections of low doses of histamine or the H_3 receptor agonist NAMH (twice a week over 12 weeks) reduced pain intensity, frequency and length of attacks as well as painkiller use in migraine patients, relating these actions to the activation of H_3 receptors controlling the release of histamine and other neurotransmitters (Millán-Guerrero *et al.*, 2006; 2009).

Alterations in the release of glutamate and neuroactive peptides have also been linked to migraine. Higher levels of glutamate have been found in plasma and in saliva samples in migraine patients when compared with healthy controls (Tajti *et al.*, 2011), and trigeminal nociceptive fibres release the vasoactive neuropeptides, substance P, CGRP and neurokinin A (Cutrer, 2010). Pre-synaptic H_3 receptors reduce substance P and neurokinin release from sensory nerve endings as well as glutamate release in several areas of the rat brain (see Feuerstein, 2008 and Osorio-Espinoza *et al.*, 2010). Thus, the reduced efficacy of the mutant hH_3 receptor reported herein may be also related to a diminished inhibitory action of endogenous histamine on the release of glutamate and neuroactive peptides, leading to increased susceptibility to migraine attacks.

In conclusion, we have here provided evidence that a single point mutation in the third intracellular loop of the human histamine H_3 receptor modifies its functional properties. This effect may have relevance for the pathophysiology of disorders associated with the mutation, namely migraine and multiple system atrophy with orthostatic hypotension, but further research on this issue is clearly required.

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Conflicts of interest

The authors disclose no conflict of interest.

References

Aamodt AH, Stovner LJ, Langhammer A, Hagen K, Zwart JA (2007). Is headache related to asthma, hay fever, and chronic bronchitis? The Head-HUNT study. Headache 47: 204–212.

Alexander SP, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Arrang JM, Morisset S, Gbahou F (2007). Constitutive activity of the histamine H_3 receptor. Trends Pharmacol Sci 28: 350–357.

Bongers G, Bakker RA, Leurs R (2007). Molecular aspects of the histamine H_3 receptor. Biochem Pharmacol 73: 1195–1204.

Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22: 3099–3108.

Cogé F, Guénin SP, Audinot V, Renouard-Try A, Beauverger P, Macia C *et al.* (2001). Genomic organization and characterization of splice variants of the human histamine H_3 receptor. Biochem J 355: 279–288.

Colosimo C (2011). Nonmotor presentations of multiple system atrophy. Nat Rev Neurol 7: 295–298.

Cutrer FM (2010). Pathophysiology of migraine. Semin Neurol 30: 120–130.

DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK (2007). β-Arrestins and cell signaling. Annu Rev Physiol 69: 483–510.

Feuerstein TJ (2008). Presynaptic receptors for dopamine, histamine, and serotonin. Handb Exp Pharmacol 184: 289–338.

Flores-Clemente C, Osorio-Espinoza A, Escamilla-Sánchez J, Arias JM, Arias-Montaño JA (2012). Comparison of the pharmacological and signaling properties of wild-type and A280V mutant human histamine H_3 receptors expressed in CHO-K1 cells. Inflamm Res 61 (Suppl. 2): S77.

Gardner B, Hall DA, Strange PG (1996). Pharmacological analysis of dopamine stimulation of [^{35}S]-GTPγS binding via human D_{2short} and D_{2long} dopamine receptors expressed in recombinant cells. Br J Pharmacol 118: 1544–1550.

Gutkind JS (2000). Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. Sci STKE 2000: re1.

Guyenet PG (2006). The sympathetic control of blood pressure. Nat Rev Neurosci 7: 335–346.

Haas HL, Sergeeva OA, Selbach O (2008). Histamine in the nervous system. Physiol Rev 88: 1183–1241.

Heatley RV, Denburg JA, Bayer N, Bienenstock J (1982). Increased plasma histamine levels in migraine patients. Clin Allergy 12: 145–149.

Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR (1993). Gene splicing by overlap extension. Methods Enzymol 217: 270–279.

Ishikawa M, Watanabe T, Kudo T, Yokoyama F, Yamauchi M, Kato K *et al.* (2010). Investigation of the histamine H_3 receptor binding site. Design and synthesis of hybrid agonists with a lipophilic side chain. J Med Chem 53: 6445–6456.

Jecmenica-Lukic M, Poewe W, Tolosa E, Wenning GK (2012). Premotor signs and symptoms of multiple system atrophy. Lancet Neurol 11: 361–368.

Kim SK, Fristrup P, Abrol R, Goddard WA 3rd (2011). Structure-based prediction of subtype selectivity of histamine H_3 receptor selective antagonists in clinical trials. J Chem Inf Model 51: 3262–3274.

Koyama M, Seyedi N, Fung-Leung WP, Lovenberg TW, Levi R (2003). Norepinephrine release from the ischemic heart is greatly enhanced in mice lacking histamine H_3 receptors. Mol Pharmacol 63: 378–382.

Ku M, Silverman B, Prifti N, Ying W, Persaud Y, Schneider A (2006). Prevalence of migraine headaches in patients with allergic rhinitis. Ann Allergy Asthma Immunol 97: 226–230.

Leurs R, Bakker RA, Timmerman H, de Esch IJ (2005). The histamine H_3 receptor: from gene cloning to H_3 receptor drugs. Nat Rev Drug Discov 4: 107–120.

Ligneau X, Morisset S, Tardivel-Lacombe J, Gbahou F, Ganellin CR, Stark H *et al.* (2000). Distinct pharmacology of rat and human histamine H_3 receptors: role of two amino acids in the third transmembrane domain. Br J Pharmacol 131: 1247–1250.

Lovenberg TW, Roland BL, Wilson SJ, Jiang X, Pyati J, Huvar A *et al.* (1999). Cloning and functional expression of the human histamine H_3 receptor. Mol Pharmacol 55: 1101–1107.

Millán-Guerrero RO, Isais-Millán R, Benjamín TH, Tene CE (2006). N^{α}-methyl histamine safety and efficacy in migraine prophylaxis: phase III study. Can J Neurol Sci 33: 195–199.

Millán-Guerrero RO, Isais-Millán S, Barreto-Vizcaíno S, Rivera-Castaño L, Rios-Madariaga C (2009). Subcutaneous histamine versus botulinum toxin type A in migraine prophylaxis: a randomized, double-blind study. Eur J Neurol 16: 88–94.

Millán-Guerrero RO, Baltazar-Rodríguez LM, Cárdenas-Rojas MI, Ramírez-Flores M, Isais-Millán S, Delgado-Enciso I *et al.* (2011). A280V polymorphism in the histamine H_3 receptor as a risk factor for migraine. Arch Med Res 42: 44–47.

Oldham WM, Hamm HE (2007). How do receptors activate G proteins? Adv Protein Chem 74: 67–93.

Osorio-Espinoza A, Ramos-Jimenez J, Arias-Montaño JA (2010). Presynaptic control by histamine H_3 receptors of neurotransmitter release. In: Shahid M, Khardori N, Kahan RA, Tripathi T (eds). Biomedical Aspects of Histamine: Current Perspectives. Springer: London, pp. 339–370.

Osorio-Espinoza A, Alatorre A, Ramos-Jimenez J, Garduño-Torres B, García-Ramírez M, Querejeta E *et al.* (2011). Pre-synaptic histamine H_3 receptors modulate glutamatergic transmission in rat globus pallidus. Neuroscience 176: 20–31.

Pillot C, Heron A, Cochois V, Tardivel-Lacombe J, Ligneau X, Schwartz JC *et al.* (2002). A detailed mapping of the histamine H_3 receptor and its gene transcripts in rat brain. Neuroscience 114: 173–193.

Rosethorne EM, Charlton SJ (2011). Agonist-biased signaling at the histamine H_4 receptor: JNJ7777120 recruits β-arrestin without activating G proteins. Mol Pharmacol 79: 749–757.

Rouleau A, Ligneau X, Tardivel-Lacombe J, Morisset S, Gbahou F, Schwartz JC *et al.* (2002). Histamine H_3-receptor-mediated [^{35}S]GTPγ[S] binding: evidence for constitutive activity of the recombinant and native rat and human H_3 receptors. Br J Pharmacol 135: 383–392.

Silver RB, Poonwasi KS, Seyedi N, Wilson SJ, Lovenberg TW, Levi R (2002). Decreased intracellular calcium mediates the histamine H_3-receptor-induced attenuation of norepinephrine exocytosis from cardiac sympathetic nerve endings. Proc Natl Acad Sci USA 99: 501–506.

Tajti J, Párdutz A, Vámos E, Tuka B, Kuris A, Bohár Z *et al.* (2011). Migraine is a neuronal disease. J Neural Transm 118: 511–524.

Uveges AJ, Kowal D, Zhang Y, Spangler TB, Dunlop J, Semus S *et al.* (2002). The role of transmembrane helix 5 in agonist binding to the human H_3 receptor. J Pharmacol Exp Ther 301: 451–458.

Wess J (1998). Molecular basis of receptor/G-protein-coupling selectivity. Pharmacol Ther 80: 231–264.

Wiedemann P, Bönisch H, Oerters F, Brüss M (2002). Structure of the human histamine H_3 receptor gene (HRH_3) and identification of naturally occurring variations. J Neural Transm 109: 443–453.

Zamyatnin AA (1972). Protein volume in solution. Prog Biophys Mol Biol 24: 107–123.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Location of the A280V mutation in the human H_3R_{445}. A cytosine to thymine transition at nucleotide position 839 with respect to the start codon results in an alanine to valine exchange at amino acid position 280 in the third intracellular loop (red circle).

Figure S2 Characteristics of forskolin-induced cAMP accumulation and hH_3 receptor-mediated inhibition. In all determinations cells were pre-incubated (15 min) with 1 mM IBMX and then exposed for 15 min to forskolin. (A,B) Concentration-response curve in CHO-K1 cells. (A) Representative experiment. Values are means ± SEM from four replicates. (B) Analysis of the concentration–response curve from four experiments. Values are expressed as pmol cAMP per sample after subtraction of basal accumulation, and correspond to the means ± SEM. Lines are data best-fit by linear regression analysis. (C,D) Representative determinations of H_3 receptor-mediated inhibition of forskolin-induced cAMP accumulation in CHO-K1-hH_3R_{WT} (C) or CHO-K1-hH_3R_{A280V} (D) cells. Cells were incubated for 15 min with forskolin (10 μM) in the absence and presence of the H_3 receptor agonist RAMH, added 5 min before forskolin. Values are means ± SEM from three to four replicates from representative determinations. The experiments were repeated a further 4 times with similar results.

Figure S3 Representative determinations for [^{35}S]-GTPγS binding. Membranes from CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells were incubated (30 min) with 50 pM [^{35}S]-GTPγS in the presence and absence of the indicated drug concentrations. (A) Stimulation by the agonist RAMH. (B) Inhibition by the inverse agonist ciproxifan. For both panels values are means ± SEM from four replicates from a representative experiment after subtraction of non-specific binding. The line drawn is the best fit to a logistic equation. The experiment was repeated a further four times with similar results.

Figure S4 Effect of H_3 receptor activation on ERK1/2 phosphorylation. ERK1/2 phosphorylation was analysed by Western blot. (A) Time course. CHO-K1-hH_3R_{WT} or CHO-K1-hH_3R_{A280V} cells were incubated for 1–15 min with the agonist RAMH (100 nM). A1) Representative blot. Similar results were obtained in two independent determinations for each cell line. P-ERK1/2, phosphorylated ERK1/2. A2) Densitometric analysis. Western blot target bands were quantified by densitometry analysis and normalized according to the intensity of the non-phosphorylated ERK1/2 signal on the same lane. Values are expressed as percentage of basal phosphorylation and correspond to the means from two determinations. (B) Concentration-response curves. Cells were incubated for 5 min with the indicated concentrations of the agonist RAMH. B1) Representative blot. Similar results were obtained in three other independent experiments. B2) Analysis from four determinations. Values (means ± SEM) are expressed as percentage of basal phosphorylation. The lines drawn are the best fit to a logistic equation. Estimates for maximum stimulation and EC_{50} are given in the text.