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Domain swapping in the human histamine H_1 receptor[§¥].

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TM	transmembrane
tr-FRET	time-resolved fluorescence resonance energy transfer
GPCRs	G-protein coupled receptors

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Abstract

G-protein coupled receptors (GPCRs) represent the largest family of receptors involved in transmembrane signalling. Although these receptors were generally believed to be monomeric entities, accumulating evidence supports the presence of GPCRs in multimeric forms. Here, using immunoprecipitation as well as time-resolved fluorescence resonance energy transfer (tr-FRET) to assess protein-protein interactions in living cells, we unambiguously demonstrate the occurrence of dimerisation of the human histamine H_1 receptor. We also show the presence of domain swapped H_1 receptor dimers in which there is the reciprocal exchange of TM domains 6 and 7 between the receptors present in the dimer. Mutation of aspartate^{107} in transmembrane domain (TM) 3 or phenylalanine^{432} in TM6 to alanine results in two radioligand-binding deficient mutant H_1 receptors. Co-expression of H_1 $D^{107}A$ and H_1 $F^{432}A$, however, results in a reconstituted radioligand binding site that exhibits a pharmacological profile that corresponds to the wild-type H_1 receptor. Interestingly, the H_1 receptor radioligands [^{3}H]mepyramine and [^{3}H]-(-)-*trans*-H_2-PAT show differential saturation binding values (B_{max}) for wild-type H_1 receptors, but not for the radioligand binding site that is formed upon co-expression of H_1 $D^{107}A$ and H_1 $F^{432}A$ receptors, suggesting the presence of different H_1 receptor populations.

Introduction

GPCRs play an important role in the transduction of extracellular signals across the cellular plasma membrane by the specific recognition and binding of chemically diverse ligands, such as photons, ions, neurotransmitters, and peptides. As such, these receptors are involved in the regulation of a variety of important (patho)physiological processes, including vision, taste and olfaction, emotions and cognition, allergy and immune regulation (Rockman et al., 2002).

Despite the important role of GPCRs in physiology and their position as important drug targets, detailed insight regarding molecular mechanisms of drug-GPCR interaction are still lacking. This is well illustrated by the fact that until recently GPCRs were thought to act as monomeric transmembrane entities (Devi, 2001). Accumulating evidence, using a variety of techniques, now suggests that various GPCRs exist in monomeric, dimeric, and various oligomeric forms (Maggio et al., 1999; Gomes et al., 2000; Dean et al., 2001). The existence of homodimers has been shown for e.g. β_2-adrenergic receptors (Hebert et al., 1996; Angers et al., 2000; Jordan et al., 2001), κ- and δ-opioid receptors (Cvejic and Devi, 1997; Gomes et al., 2000), metabotropic glutamate receptor 5 (Robbins et al., 1999), calcium-sensing receptor (Bai et al., 1998; Pace et al., 1999; Zhang et al., 2001), M_3 muscarinic receptor (Maggio et al., 1993a; Zeng and Wess, 1999), vasopressin V2-receptor (Zhu and Wess, 1998), and various somatostatin receptors (Rocheville et al., 2000; Pfeiffer et al., 2002), and dopamine receptor subtypes (Nimchinsky et al., 1997; Zawarynski et al., 1998; Gines et al., 2000).

The human histamine H_1 receptor (H_1R) is a prototypic GPCR that is an important target for pharmaceutical drug development. For many years H_1R antagonists (also often referred to as 'antihistamines') have been successfully used for the treatment of a variety of allergic conditions (Woosley, 1996). We previously have successfully identified the H_1R binding site for prototypic H_1R antagonists (Wieland et al., 1999) and have reclassified various clinically used H_1R antagonists as inverse H_1R agonists on the basis of their ability to inhibit spontaneous H_1R activity (Bakker et al., 2000). In view of the emerging concept of GPCR dimerisation we have now investigated the potential oligomerisation of the H_1R by utilising three techniques: immunoprecipitation of N-terminally epitope-tagged receptors followed by immunodetection of the tagged receptors, time-resolved FRET, using a combination of fluorescently labelled antibodies recognizing N-terminally epitope-tagged receptors, as well as the complementation of radioligand binding-sites upon co-expression of two distinct mutant receptors for which the individual mutations render the receptors radioligand insensitive. Our studies show that the histamine H_1 receptor is constitutively expressed as both monomeric and multimeric entities. Moreover, as previously suggested (Booth et al., 1999; Bucholtz et al., 1999; Choksi et al., 2000; Booth et al., 2002), our studies indicate that the recently introduced H_1 receptor radioligand [^{3}H]-(-)-*trans*-H_2-PAT specifically binds to only a subpopulation of multimeric H_1Rs. These data shed a new light on the mechanism of drug receptor interaction at this therapeutically relevant target.

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Methods

Materials

Cell culture media, LipofectAMINE, penicillin, and streptomycin were obtained from Life Technologies (Merelbeke, Belgium), and Calf serum was obtained from (Integro BV, Dieren, the Netherlands). [^{3}H]mepyramine (20 Ci/mmol) was purchased from NEN (Zaventem, Belgium). Bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine (2-[4-imidazolyl]ethylamine hydrochloride), mepyramine (pyrilamine maleate), and polyethyleneimine were purchased from Sigma RBI (USA). The H_1 receptor radioligand [^{3}H]-(-)-*trans*-H_2-PAT ([^{3}H]-(-)-*trans*-1-phenyl-3-*N*,*N*-dimethylamino-1,2,3,4-tetrahydronaphthalene; 85 Ci/mmol) was synthesised as described (Wyrick et al., 1994; Booth et al., 2002). Gifts of mianserin hydrochloride (Organon NV, The Netherlands), and *(R)*- and *(S)*-cetirizine hydrochloride (UCB Pharma, Belgium), the expression vector pcDEF$_3$ (Goldman et al., 1996) (Dr. J. Langer), and of the cDNAs encoding the human H_1R (Fujimoto et al., 1999) (Dr. H. Fukui), the mutant human H_1R Asp^{107}Ala (Ohta et al., 1994; Moguilevsky et al., 1998) (UCB Pharma, Belgium), and the mutant human H_3R Asp^{114}Glu (Uveges et al., 2002) (Wyeth, USA) are greatly acknowledged.

Site-directed mutagenesis

The mutant human H_1 receptor Phe^{432}Ala was created by Altered Sites' II (Promega) according to the manufacturers' protocol. All mutant receptors were sub-cloned into the expression vector pcDEF$_3$, and verified by sequencing.

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Cell culture and transfection

COS-7 African green monkey kidney cells were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin and 5 % (v/v) FCS. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described (Wieland et al., 1999). The total amount of the transfected plasmid DNA was maintained constant by addition of $pcDEF_3$.

HEK293 cells were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere in DMEM containing 2 mM L-glutamine, supplemented with 10 % (v/v) newborn calf serum. Cells were grown to 60-80 % confluency before transient tranfection in 100 mm dishes. Transfection was performed using LipofectAMINE reagent, according to the manufacturer's instructions.

Histamine H_1-receptor binding studies

Cells used for radioligand binding-studies were harvested 48 h after transfection and homogenised in ice-cold H_1R binding buffer (50 mM Na_2/K-phosphate buffer (pH 7.4)). For saturation isotherms cell membrane homogenates were incubated for 30 minutes at 30 °C with 0.01 to 17.0 nM [^{3}H]mepyramine or for 60 minutes at 30 °C with 0.01 to 5.8 nM [^{3}H]-(–)-*trans*-H_2-PAT, in a total assay volume of either 400 µL, or 100 µL buffer, respectively. For competition binding assays the cell homogenates were incubated for 30 min at 30 ºC with 0.1-10,000 nM test ligand in the presence of ~7 nM [^{3}H]mepyramine, or for 60 minutes at 30 °C with ~1nM [^{3}H]-(–)-*trans*-H_2-PAT in a total volume of 400 µL

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or 100 µL buffer, respectively. The non-specific binding was determined in the presence of 1 µM mianserin for both radioligands. The incubations were stopped by rapid dilution with 3 mL ice-cold H_1R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3mL buffer and radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a non-linear, least squares curve-fitting procedure using Graphpad Prism® (GraphPad Software Inc., San Diego, CA). Protein concentrations were determined according to Bradford (Bradford, 1976), using BSA as a standard. All data are represented as mean ± S.E.M from at least three independent experiments in triplicate. Statistical significance was determined by Student's unpaired t-test ($p < 0.05$ was considered statistically significant).

Time-resolved FRET

The experiments were conducted as described previously (McVey et al., 2001; Carrillo et al., 2003). Briefly, HEK293 cells were transfected with cDNA encoding the human H_1R-FLAG or H_1R-c-myc or co-transfected with both constructs together. Time-resolved FRET was assessed in whole cells expressing the aforementioned receptors or in membrane homogenates from these cells. The final amount of sample per assay were 250 µg of protein in the case of cell membranes and 1.2-1.5 x 10^6 cells when using whole cells. Samples were incubated during 2 hours at room temperature in PBS (16 mM Na_2HPO_4, 5 mM NaH_2PO_4, 150 mM NaCl) containing 50% New Born Calf Serum (v/v), 5 nM of Eu^{3+}-labelled anti-c-myc (Perkin Elmer Life Sciences), and 15 nM of

allophycocyanin-labelled anti-FLAG (Cis Bio International) antibodies. After the incubation samples were washed twice with PBS, the final pellet resuspended in 50 µl of the same buffer and transferred to a 384-well microtiter plate. Energy transfer was measured by exciting the Eu^{3+} at 320 nm and monitoring the allophycocyanin emission for 1000 µs at 665 nm using a Victor^2 1420 Multilabel Counter (Perkin Elmer Life Sciences) configured for time-resolved fluorescence after a 50 µs delay.

GPCR co-immunoprecipitation studies

Co-immunoprecipitation studies using FLAG and c-myc tagged forms of the human H_1R were performed as in (McVey et al., 2001; Ramsay et al., 2002), except that 30U/ml of endoglycosidase F were added to deglycosylate the receptor.

Results

Co-immunoprecipitation of human H_1 receptors

The human H_1R was modified at the N terminus to include c-myc or FLAG epitope tags immediately after the N-terminal methionine. Following transient co-expression of both tagged forms of the receptor and immunoprecipitation with anti-FLAG antibodies, the samples were resolved by SDS-PAGE and immunoblotted to detect anti-c-myc immunoreactivity. Two bands of an apparent molecular mass of 48 and 100 kDa were observed, which would be consistent with the detection of both monomeric and dimeric forms of the H_1 receptor (Figure 1A). Other bands of higher molecular mass were also detected, which could be due to either the presence of higher order oligomers or receptor aggregation during the denaturalisation of the samples prior to SDS-PAGE. Furthermore, the anti-c-myc-immunoreactivity was detected neither when the c-myc and FLAG tagged forms of the receptor were expressed in separate cell populations nor when these two forms of the receptor were expressed independently and mixed before immunoprecipitation. Thus, the formation of aggregates during the solubilisation process can be ruled out. In order to confirm appropriate expression of both tagged forms of the receptor, samples of the cell lysates were also immunoblotted with anti-c-myc and anti-FLAG antibodies (Figure 1B).

Time-resolved Fluorescent Resonance Energy Transfer

Time-resolved FRET fluorescence results (665 nm emission after excitation at 320 nm) obtained with the different samples are shown in Figure 2. In both cases, whole and cell

membranes, there is a clear specific signal when comparing the results observed for cells co-expressing both epitope-tagged receptors in relation to the ones resulting from a mixture of membranes or cells individually expressing each of those receptors. This FRET signal can only be explained due to the resonance energy transfer from anti-c-myc-Eu^{3+} antibodies bound to H_1R-c-myc receptors to anti-FLAG-allophycocyanin antibodies bound to H_1R-FLAG receptors. Since this resonance energy transfer can only take place within 10 nm, these date indicate the formation of H_1R multimers in living cells.

Similar results were obtained for FLAG-tagged gpH_1 receptors expressed in CHO cells using the experimental assay conditions according to Gazi and coworkers (Gazi et al., 2003), indicating also the gpH_1 receptors form multimers in living cells.

[^{3}H]mepyramine binding studies to mutant H_1 receptors

In our study using human H_1R mutants in which either Asp^{107} or Phe^{432} is changed into alanine, we confirm (Figure 3A) previous studies reporting the loss of [^{3}H]mepyramine binding to guinea-pig H_1Rs in which the corresponding amino acids (Asp^{116} and Phe^{432}) were changed to alanine (Wieland et al., 1999). Moreover, we observed that transient co-expression of both H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutants resulted in reconstitution of [^{3}H]mepyramine binding-sites. Furthermore, the reconstitution of [^{3}H]mepyramine binding was only demonstrated when the H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutants were co-transfected. Physical mixing of the membranes, expressing H_1 Asp^{107}Ala, or H_1 Phe^{432}Ala receptors, prior to [^{3}H]mepyramine binding, did not lead to significant binding (Figure 3A). In parallel, co-transfection of a mutant human histamine H_3 receptor, H_3 Asp^{114}Glu, in combination with the H_1 Phe^{432}Ala mutant, did not result in significant

[^{3}H]mepyramine binding (Figure 3A). A detailed characterisation of the [^{3}H]mepyramine binding-sites that are formed upon co-expression of H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors by saturation analysis revealed a K_D for [^{3}H]mepyramine of 1,8 ± 0,1 nM (Figure 3B). Although the K_D value of [^{3}H]mepyramine for the reconstituted binding-site is in agreement with its K_D value for the wild-type human H_1 receptor (K_D = 1,2 ± 0,1 nM), the number of binding-sites is greatly reduced (see Table 1). A more detailed investigation of the pharmacological profiles of the [^{3}H]mepyramine binding-sites that are formed upon co-expression of both H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutant receptors revealed a clear human H_1R profile of these reconstituted [^{3}H]mepyramine binding-sites (Figure 3C and Table 2), including the known stereoselectivity for the enantiomers of cetirizine (Bakker et al., 2000). The difference in affinity of mepyramine for the wild-type H_1R and for the reconstituted [^{3}H]mepyramine binding-site that is formed upon co-expression of both H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutant receptors may result from Hill coefficients (n_H) that deviate from unity (Lazareno and Birdsall, 1993). However, the Hill coefficients for mepyramine displacing [^{3}H]mepyramine from either wild-type H_1Rs or from the reconstituted binding sites do not deviate from unity (n_H= -0,9 ± 0,1, and -1,1 ± 0,1, respectively; see also Table 2).

[^{3}H]-(-)-trans-H_2-PAT binding studies to mutant H_1 receptors

Previous findings suggests that the recently described H_1R radioligand [^{3}H]-(-)-*trans*-H_2-PAT, might selectively label H_1R dimers (Booth et al., 2002). In agreement with the previously reported binding-characteristics of [^{3}H]-(-)-*trans*-H_2-PAT to rat and guinea-pig H_1Rs (Booth et al., 1999; Bucholtz et al., 1999; Choksi et al., 2000; Booth et al.,

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2002), we observed a high affinity [^{3}H]-(-)-*trans*-H_2-PAT binding-site in COS-7 cells expressing the wild-type human H_1R (K_D = 1,2 ± 0,4 nM) and a significant lower number of [^{3}H]-(-)-*trans*-H_2-PAT binding-sites (B_{max} = 3,4 ± 1,0 pmol/mg protein) in comparison to the number of [^{3}H]mepyramine binding sites that can be detected in the same preparation (Table 1).

Measuring [^{3}H]-(-)-*trans*-H_2-PAT binding to COS-7 cells membranes expressing the mutant human H_1 Asp^{107}Ala or H_1 Phe^{432}Ala receptors individually, did not result in any significant specific binding (<25 dpm, see also Figure 4A). However, upon co-expression of both mutant receptors, we observed the specific reconstitution of [^{3}H]-(-)-*trans*-H_2-PAT binding-sites (Figure 4). The reconstitution of [^{3}H]-(-)-*trans*-H_2-PAT binding-sites was only observed upon co-expression of H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutant H_1Rs, physical mixing of membranes of cells individually expressing either H_1 Asp^{107}Ala, or H_1 Phe^{432}Ala receptors, prior to radioligand binding experiments, did not lead to the reconstitution of [^{3}H]-(-)-*trans*-H_2-PAT binding-sites. A detailed characterisation of the [^{3}H]-(-)-*trans*-H_2-PAT binding-sites that are formed upon co-expression of H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors by saturation analysis revealed a K_D for [^{3}H]-(-)-*trans*-H_2-PAT of 3,0 ± 0,6 nM (Figure 4B and Table 1). Although the K_D value of [^{3}H]-(-)-*trans*-H_2-PAT for the reconstituted binding-sites is in agreement with its K_D value for the wild-type H_1R (K_D = 1.2 ± 0.4 nM), the number of binding-sites is greatly reduced (see Table 1). A more detailed investigation of the pharmacological profiles of the [^{3}H]-(-)-*trans*-H_2-PAT binding-sites that are formed upon co-expression of both H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutant receptors revealed a clear human H_1-receptor profile of these reconstituted [^{3}H]-(-)-*trans*-H_2-PAT binding-sites (Table 2), including the known

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stereoselectivity for the enantiomers of cetirizine (Bakker et al., 2000). However, the pharmacological profile is not identical to that of the wild-type H_1R, the difference in affinity of mepyramine for the wild-type H_1R and for the reconstituted [^{3}H]-(-)-*trans*-H_2-PAT binding-site that is formed upon co-expression of both H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutant receptors may in part result from the Hill coefficient (n_H) that deviate from unity, which may result in affinity values that differ from their actual K_d values (Lazareno and Birdsall, 1993). Whereas the Hill coefficient for mepyramine displacing [^{3}H]-(-)-*trans*-H_2-PAT from wild-type H_1Rs is close to unity (n_H= -1,1 ± 0,1) the Hill coefficient for mepyramine displacing [^{3}H]-(-)-*trans*-H_2-PAT from the reconstituted binding sites deviates from unity (n_H= -0,5 ± 0,1; see also Table 2).

These data indicate that reconstitution of radioligand binding-sites upon the co-expression H_1 Asp^{107}Ala and H_1 Phe^{432}Ala mutant H_1Rs is accompanied by the detection of a considerable lower number of radioligand binding-sites, when compared to the number of radioligand binding-sites upon expression of wild-type H_1 receptors. Moreover, although both [^{3}H]mepyramine and [^{3}H]-(-)-*trans*-H_2-PAT identify a significantly different number of binding-sites for the wild-type H_1R (Booth et al., 2002), they identify an identical number of reconstituted radioligand binding-sites that are formed upon co-expression of the two radioligand binding defective mutant human H_1Rs: H_1 Asp^{107}Ala and H_1 Phe^{432}Ala (Table 1).

Discussion

Recently, compelling evidence has emerged that GPCRs may be present as oligomers in the plasma membrane. Various receptors belonging to different GPCR subfamilies have been shown to form dimeric and/or oligomeric (Milligan, 2001; Rios et al., 2001; Gazi et al., 2002; Agnati et al., 2003). The occurrence of dimeric GPCRs has been shown using a variety of techniques, which has also resulted in the identification of several mechanisms of GPCR dimerisation. Two structural models for dimer formation have been suggested (Hebert et al., 1996): contact dimers and domain swapped dimers (Dean et al., 2001; Filizola et al., 2002; George et al., 2002). In contact dimers, certain domains of individual receptors interact, most likely through hydrophobic interactions, while maintaining their respective ligand binding domains. The domain swapped dimers are thought to form two ligand binding domains that are created upon the mutual exchange of transmembrane domains from both receptors (Maggio et al., 1993a; Gouldson et al., 2000) (see also Figure 5).

In the present study we have investigated the potential dimerisation of the human histamine H_1R. We demonstrate that immunoprecipitation of epitope-tagged H_1Rs with specific antibodies results in the detection of bands of apparent size corresponding to the estimated molecular weight of monomeric, dimeric, as well as higher order oligomeric human H_1Rs. Although these studies clearly indicate the possible formation of dimeric H_1Rs, several issues have been raised about the use of co-immunoprecipitation studies for demonstrating dimerisation of GPCRs (Milligan, 2001). Further studies will need to be performed to verify the functional expression of the higher order oligomeric forms of the

H_1R, however, we have obtained evidence for dimerisation of epitope-tagged H_1Rs using time resolved fluorescence resonance energy transfer (tr-FRET) experiments. Although these data indicate the presence of dimeric H_1Rs at the cell surface of living cells and in membrane preparations derived from these cells, the H_1R dimers detected by these techniques may represent a heterogeneous population of homodimers formed by contact dimers as well as domain swapped dimers. Receptor mutagenesis approaches have been used to demonstrate the formation of domain-swapped dimers of α_{2C}-adrenergic receptors as well as of the M_2 and M_3 muscarinic receptors and type 1 angiotensin II receptors when they were co-expressed (Maggio et al., 1993a; Maggio et al., 1993b; Monnot et al., 1996). In these studies, co-expression of two radioligand-binding defective mutant receptors reconstituted a functional radioligand binding site, suggesting the molecular association of complementary transmembrane domains of two different defective mutant receptors. Several studies have identified critical amino acid residues in the H_1R antagonist binding-pocket (Ohta et al., 1994; Moguilevsky et al., 1998; Wieland et al., 1999). Some of the mutant H_1Rs from these studies were defective in the binding of the H_1R radioligand [^{3}H]mepyramine. Since the mutations were located in different domains of the H_1R, we investigated whether the mechanism of intermolecular complementation could be observed for this bioaminergic receptor. We have thus co-expressed two radioligand binding defective mutant human H_1Rs, H_1 $Asp^{107}Ala$ (harbouring a mutation in TM3) and H_1 $Phe^{432}Ala$ (harbouring a mutation in TM6), and monitored the formation of potential domain swapped H_1R dimers by radioligand binding assays. Co-expression of H_1 $Asp^{107}Ala$ and H_1 $Phe^{432}Ala$ receptors, but not physical mixing of membranes of cells individually expressing either human H_1 $Asp^{107}Ala$ or H_1 $Phe^{432}Ala$ receptors,

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reconstituted a [^{3}H]mepyramine binding-site exhibiting a pharmacological profile closely resembling that of the wild-type H_1R, including the affinities for [^{3}H]mepyramine and the stereospecificity towards the enantiomers of cetirizine (Bakker et al., 2000). Thus, both H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors can adopt a conformation allowing them to interact with H_1R ligands. These data clearly illustrate the formation of domain swapped H_1R dimers in which there is the reciprocal exchange of TM domains 6 and 7 between the receptors present in the dimer. The expression level of the reconstituted binding-site is considerably lower than that of the wild-type receptor. However, one should consider that the H_1R dimers that are formed by domain swapping upon co-expression of the two binding-defective mutant H_1Rs is expected to yield dimeric H_1Rs of three different compositions and only one of these will contain a single complete binding-pocket, and not two as expected for the H_1R dimer that is formed by domain swapping of wild-type H_1Rs (Figure 5). Moreover, the two binding defective mutant H_1Rs may also form both monomeric as well as contact-dimeric H_1Rs, which will not bind [^{3}H]mepyramine due to their respective mutations (see also Figure 5).

In previous studies we have used [^{3}H]-(-)-*trans*-H_2-PAT as an alternative high affinity radioligand to label H_1Rs in guinea pig brain, rat brain, and human H_1Rs expressed in CHO cells (Booth et al., 1999; Choksi et al., 2000; Booth et al., 2002). The use of [^{3}H]-(-)-*trans*-H_2-PAT or [^{3}H]mepyramine as an H_1R radiotracer in displacement studies yields comparable H_1R affinities of a variety of H_1R ligands, however, [^{3}H]-(-)-*trans*-H_2-PAT consistently labels a lower number of H_1Rs than [^{3}H]mepyramine, indicating the presence of H_1R sub-populations that may be selectively recognised by certain ligands (Booth et al., 2002). Different levels of receptor expression have also been detected for muscarinic

(Lee and el-Fakahany, 1985; el-Fakahany et al., 1986; Wreggett and Wells, 1995; Park et al., 2002) and dopamine (see Armstrong and Strange, 2001, and references cited therein; Logan et al., 2001; Seeman et al., 2003) receptor binding sites when using different radioligands. These findings have been explained by the formation of receptor dimers or oligomers (Wreggett and Wells, 1995; Armstrong and Strange, 2001; Logan et al., 2001; Park et al., 2002; Seeman et al., 2003). We have suggested that the H_1R radioligand [^{3}H]-(-)-*trans*-H_2-PAT selectively labels H_1R dimers whereas [^{3}H]mepyramine labels both mono- and multi-valent forms of the H_1R (Booth et al., 2002). Consistent with these suggestions, we observe a [^{3}H]-(-)-*trans*-H_2-PAT binding site in COS-7 cells co-expressing H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors. Expression of H_1 Asp^{107}Ala or H_1 Phe^{432}Ala receptors, or physical mixing of membranes of cells individually expressing either H_1 Asp^{107}Ala or H_1 Phe^{432}Ala receptors, did not lead to the formation of [^{3}H]-(-)-*trans*-H_2-PAT binding sites. Therefore, our data indicates both [^{3}H]mepyramine and [^{3}H]-(-)-*trans*-H_2-PAT recognise a multimeric H_1R that is formed by domain swapping. Moreover, Asp^{107} and Phe^{432} are crucial residues in the H_1R for the interaction with either mepyramine (Ohta et al., 1994; Moguilevsky et al., 1998; Wieland et al., 1999) or (-)-*trans*-H_2-PAT.

Our studies with H_1Rs indicate that although [^{3}H]mepyramine and [^{3}H]-(-)-*trans*-H_2-PAT binding to wild-type receptors results in a discrepancy in the observed H_1 receptor expression level, a nearly identical number of H_1 receptors is detected using either radioligand when binding is selectively performed on domain swapped dimeric H_1 receptors. These data demonstrate that when radioligand binding experiments are

performed on one, presumably independent population of H_1 receptors, there is no discrepancy in the number of ligand binding sites. Therefore, these data confirm our previous suggestion on the notion of distinct wild-type H_1 receptor subpopulations (Booth et al., 2002).

In conclusion, we demonstrate the existence of dimeric H_1Rs, and in particular domain-swapped H_1R dimers. These domain swapped dimers form a subpopulation of H_1Rs exhibiting a classical H_1R pharmacological profile when profiled using diverse radioligands. The current results are in accord with previous findings on the binding of [^{3}H]-(-)-*trans*-H_2-PAT in mammalian brain and suggest dimeric H_1R subpopulations may also occur in the CNS. The significance of these findings for H_1 receptor function and ligand responses needs to be further evaluated.

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b) Reprint requests:

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c) Numbered footnotes

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Legends for figures

Figure 1. Co-immunoprecipitation of differentially epitope tagged forms of the human H_1-receptor: evidence for constitutive homo-oligomerization**.** *A,* HEK293 cells were transiently transfected with empty vector (lane 1), the cDNA encoding the FLAG-H_1R (lane 2), the cDNA encoding the c-Myc-H_1R (lane 3), or both cDNAs for the FLAG-H_1R and c-Myc-H_1R (lane 4). Prior to immunoprecipitation, cell lysates from the separately expressed FLAG-H_1R and the c-Myc-H_1R were physically mixed (lane 5). Cell lysates were immunoprecipitated with anti-FLAG (lanes 1-5) antibodies, the samples resolved by SDS-PAGE, and then immunoblotted with anti-c-myc. *B,* cell lysates were prepared from transiently transfected HEK293 cells, and immunoblotted with anti-FLAG antibodies, or anti-c-myc antibodies.

Figure 2. Time-resolved FRET. Upon measuring fluorescence emission at 665 nm, after excitation at 320nm, tr-FRET signal (filled bars) is seen using either membranes or cells co-expressing H_1R-FLAG and H_1R-c-myc, due to the resonance energy transfer of specific H_1R-c-myc bound to anti-c-myc-Eu^{3+} antibody to the specific H_1R-FLAG bound anti-FLAG-allophycocyanin antibody. Mixing of two populations of cells independently expressing each epitope-tagged receptor resulted in a reduced tr-FRET signal (open bars).

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Figure 3. Reconstitution of [^{3}H]mepyramine binding. *A*, [^{3}H]mepyramine binding to COS-7 cells membranes of cells transiently transfected with cDNA encoding the empty expression vector pcDEF$_3$ (mock), the cDNA encoding the H_1R Asp^{107}Ala (D^{107}A), the cDNA encoding for the H_1R Phe^{432}Ala (F^{432}A), or equal amounts of the cDNAs encoding either H_1R Asp^{107}Ala and H_1R Phe^{432}Ala receptors (D^{107}A + F^{432}A) or H_3R Asp^{114}Glu and H_1R Phe^{432}Ala receptors (D^{114}E + F^{432}A). Also shown is the binding of [^{3}H]mepyramine to a 1:1 mix of membranes of transiently transfected cells with cDNA encoding either H_1R Asp^{107}Ala or H_1R Phe^{432}Ala (D^{107}A/F^{432}A). *B*, Representative saturation isotherm of [^{3}H]mepyramine binding to COS-7 membranes, transiently co-transfected with both H_1R Asp^{107}Ala and H_1R Phe^{432}Ala (K_D = 1,8 ± 0,1 nM, B_{max} = 0,34 ± 0,1 pmol/mg protein; see also Table 1); *C*, Representative radioligand displacement curves on COS-7 membranes, transiently co-expressing both H_1R Asp^{107}Ala and H_1R Phe^{432}Ala, for histamine (■), R- (●) and S- (□) cetirizine, (-)-*trans*-H_2-PAT (▲),and mepyramine (○), see also Table 2.

Figure 4. Reconstitution of [^{3}H]-(-)-*trans*-H_2-PAT binding sites upon co-expression of H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors. *A*, Specific [^{3}H]mepyramine (open bars) [^{3}H]-(-)-*trans*-H_2-PAT (filled bars) binding, to COS-7 cells membranes, expressing either H_1 Asp^{107}Ala (D^{107}A) or H_1 Phe^{432}Ala receptors (F^{432}A), or co-expressing H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors (D^{107}A + F^{432}A); *B*, Representative saturation isotherm of [^{3}H]-(-)-*trans*-H_2-PAT binding to COS-7 membranes co-expressing H_1 Asp^{107}Ala and H_1 Phe^{432}Ala receptors (K_D = 3,0 ± 0,6 nM, B_{max} = 0,32 ± 0,1 pmol/mg protein; see also Table 1).

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Figure 5. Models of human H_1 receptor dimerisation. GPCRs were thought to function as monomers (A), however, several lines of evidence suggests GPCRs can form oligomeric structures. Dimeric forms of GPCRs may consist of contact dimers between receptor monomers (B) or of domain swapped dimers involving the reciprocal exchange of TM domains between receptors within the dimer resulting in trans-complementation (C). Different TM domains have been reported to be involved in the formation and stabilisation of contact dimers, such as an interface involving TM4-5 (Guo et al., 2003), or an interface involving TM5-6 (as indicated here) (Maggio et al., 1993a; Gouldson et al., 2000; Liang et al., 2003) or involving TM1 (Gouldson et al., 2000; Overton and Blumer, 2002; Carrillo et al., 2003; Liang et al., 2003). The TM domains harbouring the mutation in mutant H_1Rs are indicated in gray (TM3 for H_1 $D^{107}A$, and TM6 for H_1 $F^{432}A$); functional ligand binding sites (**L**) and non-functional ligand binding sites (**X**) are indicated.

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Table 1. Radioligand binding data obtained using COS-7 cell-membranes expressing either wild-type H_1Rs (WT), or co-expressing both H_1R Asp^{107}Ala and H_1R Phe^{432}Ala, using either [^{3}H]mepyramine or [^{3}H]-(-)-*trans*-H_2-PAT as a radioligand. Data shown are mean ± S.E.M of three independent experiments, each performed in triplicate.

	[^{3}H]mepyramine		[^{3}H]-(-)-*trans*-H_2-PAT	
	K_D (nM)	B_{max} [a]	K_D (nM)	B_{max} [a]
H_1R WT	1,2 ± 0,1	21 ± 4	1,2 ±0,4	3,4 ± 1,0
H_1R Asp^{107}Ala + H_1R Phe^{432}Ala	1,8 ± 0,1	0,34 ± 0,1	3,0 ± 0,6	0,32 ± 0,1

[a] pmol/mg protein.

Table 2. Pharmacological characterisation of [^{3}H]mepyramine and [^{3}H]-(-)-*trans*-H_2-PAT binding sites in wild-type H_1R expressing cells and in cells expressing both H_1R Asp^{107}Ala and H_1R Phe^{432}Ala mutant H_1Rs ($D^{107}A + F^{432}A$). The Hill coefficient (n_H) for the displacement of the radioligand binding to the reconstituted ligand binding sites that are observed upon co-expression of the mutant H_1Rs $D^{107}A$ and $F^{432}A$ are shown. Data shown are mean ± S.E.M of three independent experiments, each performed in triplicate.

	[^{3}H]mepyramine			[^{3}H]-(-)-*trans*-H_2-PAT		
	H_1R	$D^{107}A + F^{432}A$		H_1R	$D^{107}A + F^{432}A$	
	pK_i	pK_i	n_H	pK_i	pK_i	n_H
histamine	4,1±0,1	4,2±0,1	-0,8±0,1	4,1±0,1	4,5±0,1	-0,9±0,1
mepyramine	8,7±0,1	8,0±0,1	-1,1±0,1	8,1±0,1	6,3±0,1	-0,5±0,1
(-)-*trans*-H_2-PAT	8,8±0,2	8,5±0,2	-0,8±0,2	8,2±0,1	7,3±0,1	-1,0±0,1
(R)-cetirizine	7,8±0,1	7,6±0,1	-1,1±0,1	7,5±0,1	6,9±0,1	-1,0±0,1
(S)-cetirizine	6,8±0,1	6,3±0,1	-1,1±0,1	6,4±0,1	5,6±0,1	-1,2±0,1

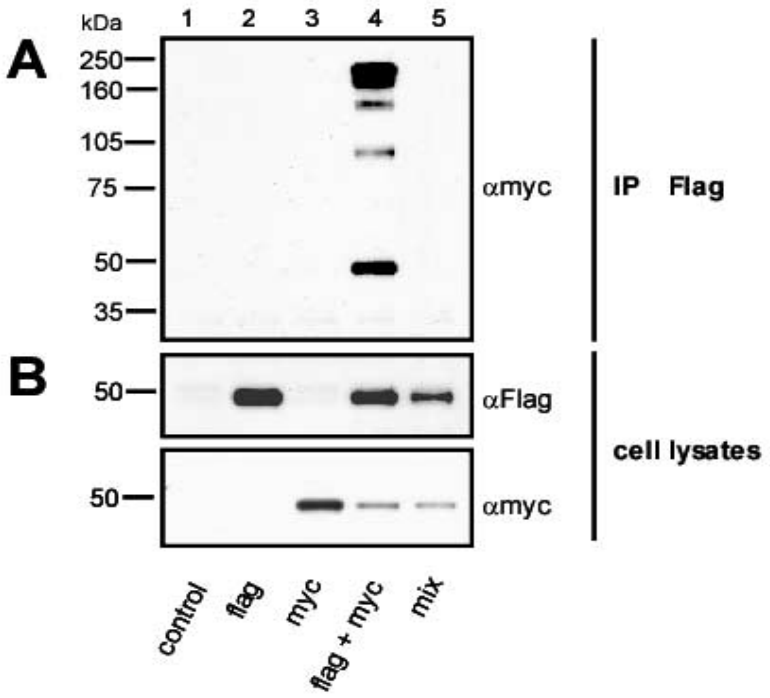

Figure 1
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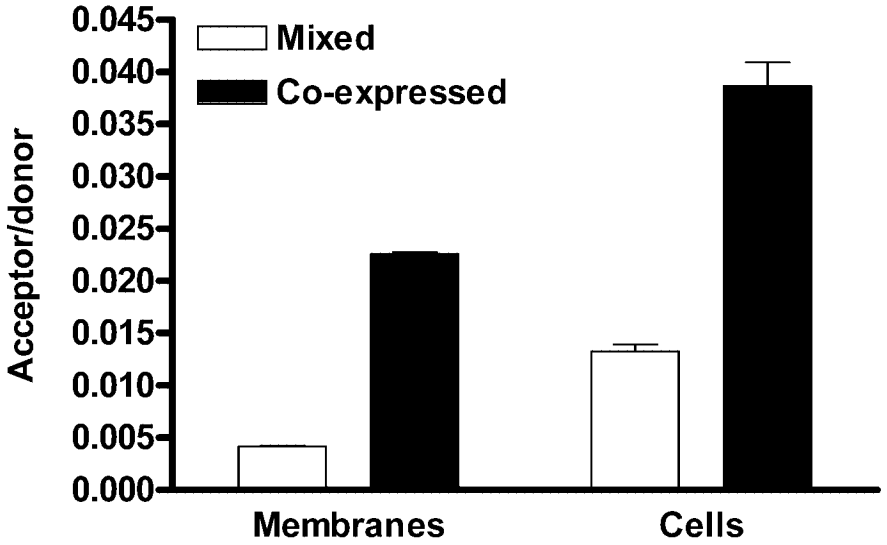

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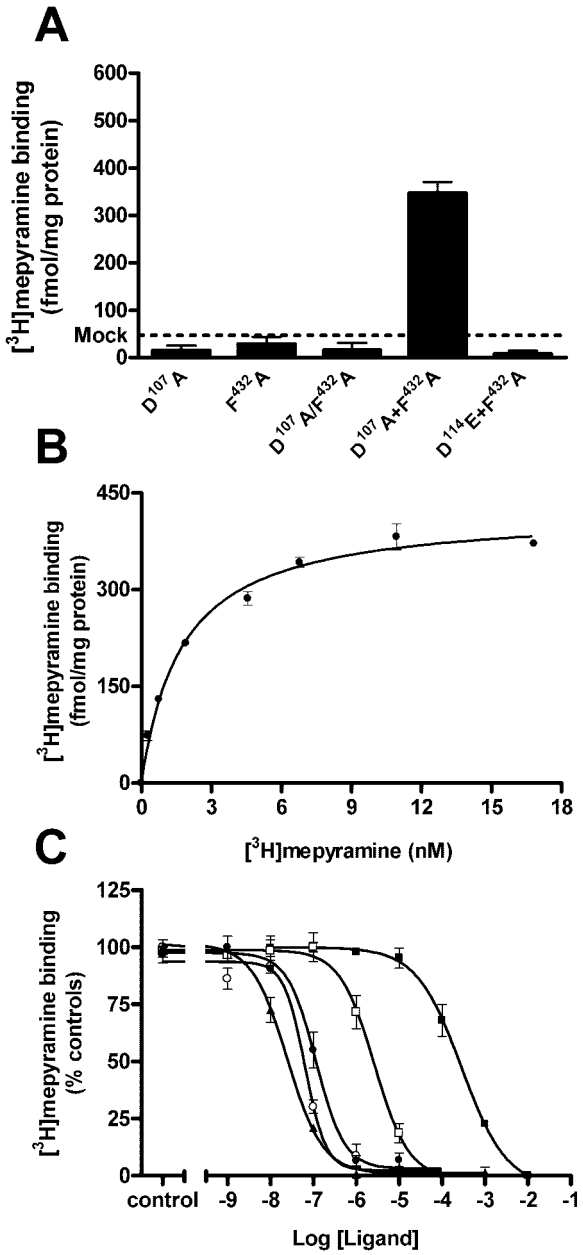

Figure 3
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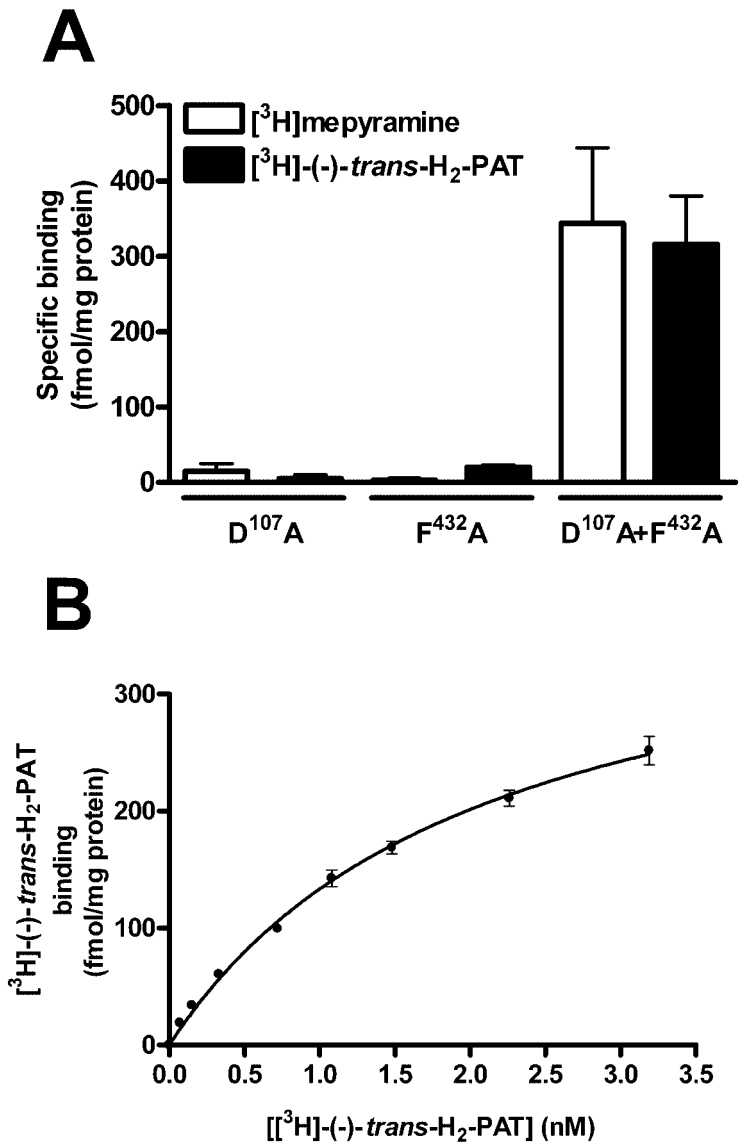

Figure 4
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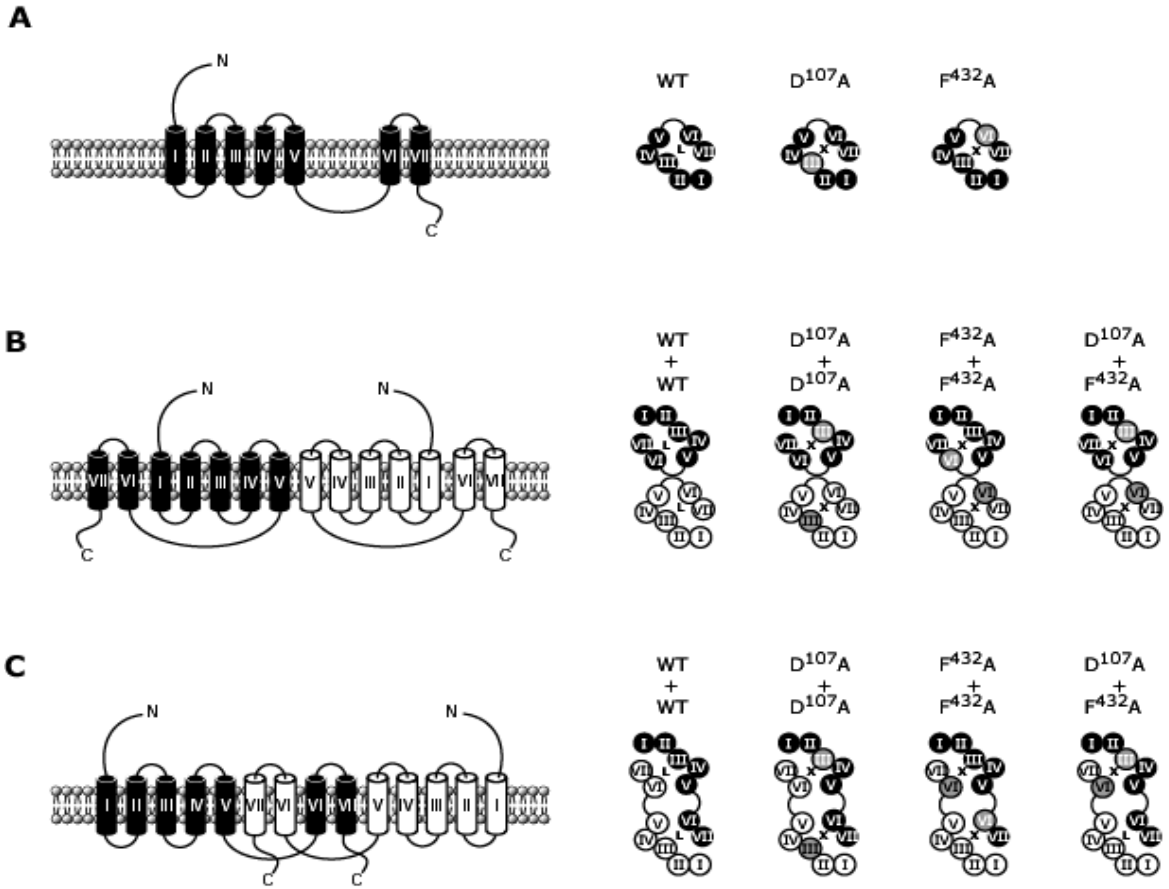

Figure 5
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