Molecular pharmacology of the human histamine $H_4$ receptor

Richard Michiel van Rijn
Molecular pharmacology of the human histamine H₄ receptor
Richard Michiel van Rijn

Printed by PrintPartners Ipskamp

Cover: Bond of union.
M.C. Escher's “Bond of Union” © 2006 The M.C. Escher Company B.V. - Baarn – Holland. All rights reserved.

Copyright © 2007 Richard Michiel van Rijn, Amsterdam. All rights reserved. No part of this thesis may be reproduced in any form or by any means without permission from the author.

Molecular pharmacology of the human histamine H₄ receptor

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Exacte Wetenschappen
op dinsdag 13 november 2007 om 13.45 uur
in het auditorium van de universiteit,
De Boelelaan 1105

door

Richard Michiel van Rijn

geboren te Abbenes
promotor: prof.dr. R. Leurs

copromotor: dr. R. A. Bakker
# Table of Contents

| Chapter 1 | Introduction | 7 |
| Chapter 2 | Evaluation of Histamine H₁-, H₂-, and H₃-Receptor Ligands at the Human Histamine H₄ Receptor: Identification of 4-Methylhistamine as the First Potent and Selective H₄ Receptor Agonist | 43 |
| Chapter 3 | Oligomerization of recombinant and endogenously expressed human histamine H₄ receptors | 67 |
| Chapter 4 | Cloning and characterization of dominant negative splice variants of the human histamine H₄ receptor | 94 |
| Chapter 5 | Mechanisms of aminergic receptor oligomerization | 119 |
| Chapter 6 | Hetero-oligomerization of the human histamine H₄ receptor and the human cytomegalovirus encoded chemokine receptor US28 | 140 |
| Chapter 7 | Overproduction of the human histamine H₄ receptor through the use of various expression systems | 159 |
| Chapter 8 | Discussion and Conclusions | 177 |
| Samenvatting | 191 |
| References | 194 |
| Abbreviations | 212 |
| Acknowledgements | 213 |
| Curriculum Vitae | 215 |
| List of publications | 216 |
1.1 G-protein coupled receptors
1.2 Histamine and histamine receptors
1.3 Scope and aim of this thesis
1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) form the largest superfamily of proteins in the human body. About 1% of the human genome encodes for over a 1000 GPCRs \(^1\). GPCRs are located at the cell surface and are expressed ubiquitously. GPCRs regulate many diverse intracellular responses and therefore are involved in all kinds of diseases, making them interesting therapeutic targets \(^2\). The name GPCRs originates from the first step in the signal transduction, which requires the direct activation of a hetero-trimeric G protein. However, this name is somewhat misleading as G-proteins are also able to couple to other proteins besides GPCRs and GPCRs are able to signal without G-proteins \(^3\). Therefore, GPCRs are sometimes referred to as heptahelical, serpentine or 7-transmembrane (TM) receptors. The name 7-TM originates from the fact that GPCRs are transmembrane proteins, which cross the membrane seven times. Each individual TM domain is an \(\alpha\)-helix connected to the next TM domain via alternating intracellular or extracellular loops, starting with an extracellular amino-terminal tail and ending with an intracellular carboxyl-terminal tail (Figure 1.1).

Figure 1.1: Computer model of a G protein- coupled receptor monomer or dimer interacting with one hetero-trimeric g-protein, based on the crystal structure of rhodopsin. Figure adapted from \(^4\)
The superfamily of GPCRs can be activated by a plethora of different stimuli, including protein hormones, lipids, peptides, biogenic amines and nucleotides but also stimuli such as tastes, odors and light. The bundle of 7 TM domains forms a cavity that is big enough to fit small ligands, such as the biogenic amines whereas larger ligands, like peptides and hormones generally interact and bind to the N-terminal tail and/or extracellular loops.

Numerous GPCRs are involved in a wide variety of diseases, including asthma, hypertension, Parkinson's disease, cancer, and HIV infection. The presence of GPCRs at the cell surface makes these receptors very accessible for therapeutic drugs. Currently about 50% of all marketed drugs are targeted at GPCRs, with sales for individual drugs reaching up to several billions of US dollars per year. Although belonging to one superfamily, GPCRs are diverse and can be subdivided into smaller families on basis of sequence homology and/or pharmacological characteristics. In general GPCRs are divided into three major subfamilies; the subfamily of rhodopsin receptor-like GPCRs (family A), secretin receptor-like GPCRs (family B) and the metabotropic neurotransmitter receptor-like GPCRs (family C). The yeast pheromone (family D), the cAMP receptors (family E) and the frizzled/smoothened family make up three minor subfamilies. Family A GPCRs form the largest of the three major subfamilies and include, among others, the aminergic receptors (histaminergic, serotonergic, muscarinergic, dopaminergic and adrenergic receptors), peptide, (rhod)opsin as well as the large subfamily of olfactory receptors.

Family B consists of approximately 25 GPCRs. These GPCRs are recognized by a range of neuropeptides and peptide hormones, like glucagon, calcitonin and secretin. One of the hallmark features of family B GPCRs is the large extracellular amino terminal tail containing numerous cysteines, which probably forms a network of disulfide bridges. Unlike family A GPCRs, no DRY motif, which is involved in the activation of family A GPCRs, is present in family B receptors.

Hallmark features for family C receptors include a short and highly conserved intracellular loop (IL3), a relatively long carboxyl terminal tail and an exceptionally long amino terminal tail, which is shaped like a Venus-fly trap to
facilitate ligand binding. Family C receptors include the metabotropic glutamate, γ-aminobutyric acid (GABA), calcium and taste receptors. The human genome project has revealed the existence and position of all GPCRs. However for several GPCRs their respective cognate ligand is unknown. These GPCRs are known as orphan GPCRs. Excluding the olfactory GPCRs, approximately 300 full open reading frames (ORFs) have been identified to encode putative GPCRs. Out of these 300, about 100 are considered orphan receptors. Much effort is invested in attempts to de-orphanize these receptors and trying to link these GPCRs to possible diseases. In addition to these orphan receptors, many genes encoding GPCRs can be alternatively spliced, adding to the complexity and magnitude of this superfamily.

**G protein coupling**

As mentioned before GPCRs primarily transduce their signals through coupling and activation of intracellular hetero-trimeric G-proteins. These G-proteins consist of three subunits; the Ga, Gβ and Gγ subunits. The Ga subunit harbours an intrinsic GTPase activity able to hydrolyze GTP to GDP. The Gβ and Gγ subunits form dimeric Gβγ-subunit that associate and dissociate from the Ga subunit depending on whether the Ga subunit is bound to GDP or GTP, respectively. In its resting state the Ga subunit is bound to GDP and is associated to a hetero-dimeric Gβγ-subunit. While bound to the Ga subunit the Gβγ-subunit inhibits the spontaneous release of GDP from the Ga subunit. Upon agonist-binding to the receptor, the activated receptor subsequently activates the intracellular hetero-trimeric G protein by causing an increase in the rate of GDP-GTP exchange on the Ga subunit. Upon activation the now GTP-bound Ga subunit dissociates from the Gβγ-subunit, and both the Ga- and Gβγ-subunit subsequently activate their respective effectors. Hydrolysis of GTP to GDP by the intrinsic GTPase returns the Ga subunit to its inactive, GDP-bound state, followed by re-association with a Gβγ-subunit. Currently 20 α subunits, 6 β subunits and 12 γ subunits have been identified, however, only four main classes of hetero-trimeric G-proteins can be
distinguished: $\alpha_s$, $\alpha_i$, $\alpha_q$, $\alpha_{12}$. The $\alpha_s$- and $\alpha_i$-proteins stimulate or inhibit the activity of adenyl cyclase, to result in an increase or decrease in cAMP formation, respectively. The cAMP subsequently can activate protein kinase A (PKA). $\alpha_q$-proteins activate phospholipase C, which lead to the hydrolysis of phosphatidylinositol biphosphate (PIP$_2$), resulting in the formation of diacyl glycerol (DAG) and inositol triphosphate (InsP$_3$), subsequently leading to activation of protein kinase C (PKC) and increases in intracellular Ca$^{2+}$-ions. $\alpha_{12}/\alpha_{13}$-proteins can regulate the small G-protein RhoA (Figure 1.2).

Figure 1.2: Overview of G-protein coupling and signaling pathways mediated through GPCR activation. GPCRs can be stimulated by a wide assortment of ligands like biogenic amines, ions, lipids and hormones. Activated GPCRs can transfer the extracellular stimulus into intracellular signals through binding to a variety of G proteins ($\alpha_s$, $\alpha_o$, $\alpha_q$ and $\alpha_{12}$), which can activate intracellular and nuclear effectors. Figure adapted from 17.

Originally receptors were thought to interact with only one type of heterotrimeric G-protein. However, it is now appreciated that depending on cellular and external conditions GPCRs can promiscuously interact and signal via multiple pathways. Furthermore, recently ligands were discovered that act as agonist in systems devoid of constitutive activity, but become inverse agonists in constitutively active systems, these ligands are known as protean ligands.
In addition, GPCRs can also activate small monomeric G-proteins like Ras\(^\text{19}\). The G\(\beta\gamma\)-dimer can separately activate several different signaling pathways, such as adenyl cyclases, PLC-\(\beta\), a multitude of kinases as well as ion channels\(^\text{17,20}\) (Figure 1.3). Moreover, GPCRs can signal independently of G-proteins. This signaling is mediated through by interacting with \(\beta\)-arrestins and G-protein kinases (GRK), and can result in activation of cytoplasmic effectors such as Src, Ras and ERK, ultimately playing roles in chemotaxis and apoptosis\(^ \text{21,22}\). In conclusion, through the years increasing knowledge on GPCR signal transduction has led to a better understanding of GPCR signaling but has also revealed more of the actual complexity of GPCR signaling.

**Figure 1.3:** Overview of the current GRK- and \(\beta\)-arrestin-mediated signaling functions and biological activities. Upon agonist stimulation of a GPCR, \(\beta\)-arrestins and GRKs are recruited and activated. They subsequently activate or inhibit various signaling pathways. Additionally, \(\beta\)-arrestin 1 can translocate to the nucleus and regulate the expression of various genes through reorganization of chromatin. Adapted from\(^ \text{22}\)
Constitutive activity of GPCRs

In 1989 Costa and Herz reported for the first time on the ligand independent intrinsic basal activity of GPCRs. This observation was followed by the discovery that single point mutations were able to increase this apparent constitutive activity of GPCRs. The concept of constitutive activity has now been firmly established and has been shown to occur for over 60 GPCRs. The discovery of constitutive active GPCR was accompanied by the finding that certain ligands formerly known as antagonists were actually able to inhibit the constitutive receptor activity. In contrast to agonist, these ligands possess a negative intrinsic activity and are now known as inverse agonists. Constitutive active GPCRs may be the cause of several diseases, such as male precocious puberty, dwarfism, Kaposi’s sarcoma and congenital night blindness. The detection of constitutive activity also had implications for the ternary model describing receptor activation.

The two state model portrayed only the binding of an agonist (A) to the receptor (R), causing the activation of the formed complex (AR). The ternary model also took the G-protein (G) coupling into account, which would couple to the activated AR complex (AR'), forming the ternary AR'G complex. Constitutive activity demanded an extension of this ternary model, allowing for receptors to be activated and bind G proteins in the absence of agonists. An even more complex, but thermodynamically more complete model is the cubic ternary model (Figure 1.4). This model also allows for the formation of a non-signaling complex between the inactive receptor and the G-protein. Monczor and co-workers found evidence to support the cubic ternary complex, using the H₂ inverse agonist tiotidine. The discovery that GPCRs can form oligomeric structures, which exhibit different affinities for certain G-proteins, will undoubtedly result in models with even higher levels of complexity. An example of such a model has been described by Durroux.
Figure 1.4: Models describing GPCR systems. The traditional model merely describes the binding of an agonist (A) to the receptor (R) and subsequent activation of this complex (AR). The ternary complex introduces the G-protein (G) as a third partner, that can interact with the activated agonist-receptor complex (AR*). The ternary complex was extended after the discovery of ligand-independent activation of the receptor, allowing for the formation of R*G. The cubic ternary complex makes up a thermodynamically more complete model. Figure adapted from another source.
GPCR structure

The development of therapeutic drugs acting on GPCRs is largely based on two different strategies. For a newly identified target large libraries are tested in a high throughput screening (HTS) approach. These libraries frequently consist of a selection of known drugs, a large diversity of scaffolds, and sometimes also a selection of natural occurring compounds. A more rational approach to drug design can be employed when detailed information on the target, especially data obtained by mutational of the ligand-binding pocket, is known. Rational based drug design may benefit a great deal from the elucidation of the high resolution structures of GPCRs. So far the crystallization of GPCRs other than rhodopsin, have not yielded high resolution structural data. However, using the rhodopsin structure as template, homology models of these GPCRs can be created, which are useful for rational drug design on these GPCRs.

Although all GPCRs share the same topology of a 7TM receptor, they do not necessarily share high sequence homology. However, within the large subfamily of family A GPCRs there are several amino acid residues that seem to be important for either the structural integrity or signal transduction and are conserved among these family A GPCRs. These amino acids are: an asparagine (Asn/N) in TM1, an aspartate (Asp/D) in TM2, an arginine (Arg/R) in TM3, a tryptophan (Trp/W) in TM4, and proline residues (Pro/P) in TM5, TM6 and TM7. The arginine in TM3 is part of a larger conserved motif (D/E-R-Y) called the DRY motif. This motif is supposedly involved in keeping the receptor in its inactive conformation. On top of TM3 a conserved cysteine (Cys/C) is located. This cysteine forms a disulphide bridge with a cysteine located in extracellular loop 2. The proline in TM6 is part of the larger FxxCWxP motif, whereas the proline in TM7 is part of the conserved NPxxY motif. Whereas GPCRs of family A consist of 7 alpha helices spanning the plasma membrane, the C-terminal tail also contains a region, following TM7 that can form an eight alpha helix (Figure 1.5).
Figure 1.5: Schematic representation of a GPCR depicting highly conserved amino acids and motifs within GPCRs belonging to family A. The most conserved amino acid within each transmembrane domain has been giving the number 50, according to the Ballesteros Weinstein numbering\(^\text{37}\).

Recently the study of H\(_1\) receptor activation revealed that Asn 7.45 (according to Ballesteros Weinstein numbering\(^\text{37}\)) may play a crucial role in transferring the conformational rearrangement of the receptor upon agonist binding, to receptor activation. In the case of the H\(_1\) receptor Asn 7.45 appears to function as link between the ligand binding pocket (formed by Ser 3.36, Trp 6.48 and Phe 6.52) and conformational transition of Asn 7.49, part of the NPxxY motif, which leads to GPCR activation. The amino acids in this proposed mechanism are well conserved amino-acids (~70%), within the family A GPCRs, suggesting that the observed activation mechanism may apply to other rhodopsin-like GPCRs as well\(^\text{38}\).

**GPCR oligomerization**

Initially GPCRs were thought to exist as monomeric entities that interact in a 1:1 ratio with hetero-trimeric G-proteins. Increasingly more evidence suggesting that GPCRs may exist as well as function as dimeric or even higher oligomeric
structures have emerged. Especially the development of biophysical assays to study receptor oligomerization, including techniques making use of resonance energy transfer, largely contributed to this shift in belief. For some GPCRs, like the GABA<sub>B</sub> and taste receptors oligomerization is even a prerequisite for their function. A couple of studies showed that dimeric receptors interact in a 2:1 ratio with the cognate G-protein i.e. one dimer binding only one G protein. A more comprehensive overview on GPCR oligomerization is provided in chapter 5.

**GPCR desensitization and internalization**

To regulate the responsiveness of the cell to multiple stimulation of GPCRs over time, many receptors are desensitized after ligand binding and GPCR activation. There are two variants of desensitization: heterologous and homologous desensitization. The former, also known as agonist, non-specific desensitization, involves protein kinases A (PKA) and C (PKC), while the latter, which is also known as agonist-specific desensitization involves G protein-coupled receptor kinases (GRKs) and the two non-visual arrestins (β-arrestin 2 and arrestin 3).

For heterologous desensitization, GPCRs are phosphorylated by second messenger–dependent kinases (PKA and PKC). However, PKA and PKC can also indirectly affect GPCR desensitization by phosphorylation of GRK2. After agonist binding to the receptor GRK is translocated to the membrane where it is co-localized with the receptor. The GRK can contact several GPCRs, but can only phosphorylate the receptor that is in the appropriate conformational state, i.e. R*. Phosphorylation of receptors by GRKs alone does not lead to high levels of desensitization. However, this phosphorylation increases the affinity of the arrestins to bind to the GPCR. The arrestin binding to the receptor prevents the heterotrimeric G protein binding to the receptor and results in maximal receptor desensitization of the receptor. GRK phosphorylation usually occurs at serine/threonine clusters. Several research groups have shown that mutating the serines and threonines in these clusters to alanines abolish phosphorylation and inhibit internalization.
After desensitization the receptor is sequestered and removed from the membrane. One of the mechanisms for translocation of the receptor to intracellular compartments makes use of clathrin coated vesicles. While sequestered the receptor can be dephosphorylated followed by recycling of the receptor to the membrane (resensitization). If not recycled the endosome will fuse with a lysosome and the receptor is degraded (Figure 1.6)\(^6\). Although most receptors are only internalized following ligand (agonist) binding some receptors can be found to internalize constitutively\(^6\). It has been shown that GPCRs that do not undergo agonist activated internalization, through hetero-oligomerization, can exert a dominant negative effect on GPCRs that internalize after ligand activation, retaining these GPCRs at the cell surface\(^6\). It has also been shown that GPCR hetero-dimerization can cause GPCRs to be co-internalized with another GPCR which is internalized after agonist binding\(^6\).\(^4\),\(^5\).

**Figure 1.6:** Trafficking of GPCRs. Activation of a GPCR by an agonist leads to the dissociation of \(\alpha\) and \(\beta\gamma\) subunits. The free \(\beta\) dimers recruit G-protein receptor kinases (GRK) to the receptor, where they specifically phosphorylate agonist-occupied receptors. This, in turn, leads to the recruitment of \(\beta\)-arrestin to the receptor and targets the receptor-\(\beta\)-arrestin complexes to clathrin-coated pits. The receptor is internalized into acidic endosomes and then either dephosphorylated and recycled to the cell surface or degraded. Figure adapted from\(^10\).
1.2 Histamine and histamine receptors

Histamine

The biogenic amine histamine (2-(imidazol-4-yl)ethylamine, Figure 1.7) is an endogenous substance first described in 1910. Histamine is synthesized by decarboxylation of the amino-acid L-histidine by the enzyme L-histidine decarboxylase (HDC). However, histamine can also be taken up from certain foods, such as soy products, fish and fermented vegetables. Histamine is stored in the body mainly in cytoplasmic granules within mast cells, basophils and enterochromaffin cells but is also present in the central nervous system (CNS). In the brain histamine is synthesized and stored in neurons in the tuberomammillary nucleus of the posterior hypothalamus. Also certain cells (e.g. macrophages, dendritic cells and T cells) have been identified that can release readily synthesized histamine (neo-synthesized histamine), without prior storage.

In response to various immunological and non-immunological stimuli histamine can be released in large amounts from the cytoplasmic granules. Upon release, histamine can be metabolized through two pathways: histamine can either be methylated at the $N^\alpha$ imidazole by histamine-N-methyltransferase or be oxidized by diamine oxidase to form imidazole acetaldehyde. The actions of histamine are mediated through histamine receptors of which currently four have been identified and cloned, namely the histamine H$_1$, H$_2$, H$_3$ and H$_4$ receptor. Several histamine binding proteins exist, including cytochrome P450, which can bind histamine but also other imidazole containing histaminergic ligands, such as thioperamide and clobenpropit.

\[
\begin{align*}
\beta & \alpha & N^\alpha \\
4 \text{ or } 5 & CH_2 & CH_2 & NH_2 \\
(tele) \tau & N & N & \pi (\text{pros}) \\
2 & & & \\
\end{align*}
\]

Figure 1.7 Chemical structure of histamine
The histamine $H_1$ receptor

**Gene structure and expression**

The existence of a histaminergic receptor as mediator of histamine induced effects has been appreciated for almost a century. Although not completely aware of the target many ligands have been developed that counteracted the effect of histamine. The first so called “anti-histamine” was synthesized in 1936\(^\text{72}\) and demonstrated effectiveness in protection against histamine-induced bronchospasm\(^\text{72}\). However, the histamine $H_1$ receptor ($H_1$R) was firstly introduced by Ash and Schild in 1966\(^\text{73}\), and was defined further after the discovery of the histamine $H_2$R by Black and co-workers in 1972\(^\text{74}\). The gene encoding the human $H_1$R was eventually cloned in 1993\(^\text{75,76}\). The gene encoding the $hH_1$R is located on chromosome 3 band 3p14-p21\(^\text{77}\), or 3p25\(^\text{76,78}\). The intronless gene encodes a 487 amino acid polypeptide. Correctly folded $H_1$ protein crosses the plasma-membrane seven times and contains also other hallmark motifs, such as the conserved disulphide bridge between extracellular loop 2 and 3, attributed to GPCRs. The $H_1$R consists of a remarkable long third intracellular loop, and relatively small C-amino terminal tail. While the N-amino terminal tail holds potential N-glycosylation sites, the C-terminal tail lacks cysteine residues, thus lacking possible palmitoylation sites. The $H_1$R has an apparent molecular weight of $\sim 55$ kDa\(^\text{79}\). The existence of several single nucleotide polymorphisms of the $H_1$R, which may be linked to certain diseases have been reported\(^\text{80}\). The $H_1$R is expressed ubiquitously and can be found to exert its effects in e.g. lung smooth muscle cells, vascular endothelium, the gastro-intestinal tract, the heart and the central nervous system (CNS), including the cerebellum, neocortex, hippocampus, thalamus and nucleus accumbens\(^\text{79}\). The presence of the $H_1$R in the brain explains why the first generation of anti-histamines cause sedation as primary side effect. By reducing the ability of anti-histamines to pass the blood brain barrier, the second generation of anti-histamines does not exhibit this side effect anymore.
H<sub>1</sub>R mediated signalling

Histamine H<sub>1</sub>R signalling is regulated predominantly by coupling to G<sub>q/11</sub>-proteins \(^{81}\). Stimulation of the H<sub>1</sub>R results in activation of phospholipase C, which hydrolyses phosphatidyl 4, 5-biphosphate (PIP<sub>2</sub>) generating diacylglycol (DAG) and inositol (1,4,5) triphosphate (InsP<sub>3</sub>) in the process. Activation of InsP<sub>3</sub> receptors in the endoplasmatic reticulum (ER) by InsP<sub>3</sub> regulates the subsequent release of Ca<sup>2+</sup> \(^{82}\). The released DAG can activate protein kinase C (PKC), which in turn can phosphorylate other proteins, including the H<sub>1</sub>R resulting in desensitization of the receptor \(^{83,84}\). Activation of the H<sub>1</sub>R can also activate nuclear factor κB (NFκB). This transcription factor can regulate the transcription of several genes supposed to be involved in inflammation \(^{85}\). The H<sub>1</sub>R can signal in a ligand-independent manner as well. This constitutive activity of the H<sub>1</sub>R was first reported by Bakker and co-workers \(^{86}\). While NFκB in ligand activated H<sub>1</sub>R can occur via both the G<sub>q/11</sub>- and G<sub>βγ</sub>-subunits, the constitutively activated H<sub>1</sub>R can activate NFκB only via the G<sub>βγ</sub>-subunits \(^{85}\). In addition H<sub>1</sub>R activation has been shown to activate several other signalling pathways including activation of nitric oxide (NO) synthase \(^{87}\), activation of phospholipase A<sub>2</sub> and subsequent arachidonic acid release \(^{88-90}\), stimulation of phospholipase D (PLD) \(^{91}\). Finally the H<sub>1</sub>R was found to couple to PTX sensitive G<sub>i</sub>-proteins \(^{92}\).

Specific ligands for the H<sub>1</sub>R

The development of specific agonists for the H<sub>1</sub>R has been complicated, as changes of the ethylamine side chain of histamine, the endogenous ligand, are not well tolerated \(^{93}\). However, modifications at the 2-position of the imidazole ring of histamine are acceptable and have resulted in the development of relatively selective H<sub>1</sub>R agonists, such as 2-methylhistamine \(^{93}\) and 2-(3-trifluormethyl)phenylhistamine \(^{94}\). Other options in which the imidazole ring has been replaced by a non-imidazole moiety have resulted in H<sub>1</sub>R agonists, such as 2-pyridylethylamine and 2-thiazolylethylamine \(^{95}\).
Currently the most potent and selective H1R agonist are the so called histaprodifens. These ligands combine the histamine moiety with a diphenylalkyl substituent at the 2-position. Remarkably this diphenylalkyl substituent is typically used for H1R antagonists. Apparently maintaining the histamine moiety retains H1R agonism, whilst the additional “antagonist” substituent results a better fit and subsequent higher affinity. Another potent yet non-selective H1R agonist is 8R-lisuride. This ergot alkaloid is structurally very different from the typical H1R agonists, but is known to bind dopaminergic and serotoninergic receptors as well. The scaffold of 8R-lisuride however, might serve as lead for the development of more selective H1R agonists.

Fortunately, design of the pharmaceutically more interesting H1R antagonist has been less complicated. In general these anti-histamines contain the ethylamine moiety linked to two aromatic rings as e.g. in mepyramine. However, fusion of the aromatic rings to form tricyclic ligands, such as doxepine and clozapine, is also tolerated. As mentioned before, many of these classical H1R anti-histamines cause sedative side effects, and also can antagonize muscarinic and serotoninergic receptors. This has led to the development of new H1R antagonists practically devoid of these unwanted side effects. Several of these second generation anti-histamines have become very successful therapeutics in the treatment of allergic symptoms. Drugs like cetirizine (Zyrtec®), ketotifen (Zaditor®), Loratidine (Claritin®) and fexofenadine (Allegra®) have become billion dollar selling blockbusters.

The finding of H1R constitutive signalling has not only caused a reclassification of H1R antagonists to inverse agonists, it has also led to the development of several neutral antagonist for the H1R, such as histabudifen and histapendifen.

**Physiological roles of the H1R**

Most of the physiological effects caused by an allergic reaction can be attributed to the H1R. Constriction of the airway results from contraction of smooth muscle cells in the lung due to H1R activation. Red, swollen skin and eyes are caused by H1R mediated contraction of endothelial cells and subsequent increases in vascular
permeability. Activation of H₁R in the brain can induce sedation. While this effect is often unwanted, in the United States anti-flu medicines, containing a mixture of therapeutic drugs, are sold. Often the tablets are divided in day and night tablets, where the night tablets contain first generation anti-histamines, thus inducing sleep as part of the therapeutic effect. Although normally overshadowed by the stimulatory effects of the H₂R, in human myocardium H₁R activation is associated with inhibitory effect on heart rate.

Figure 1.8: Chemical structure of several H₁R ligands
The existence of a second histamine receptor was initially proposed by Ash and Schild, based on the inability of “anti-histamines” to block histamine-mediated gastric acid secretion. It took another six years before the idea of this H2R became generally accepted. In 1972 Black and co-workers synthesized the first selective H2R antagonist burimamide, which could block histamine effects in stomach and heart, but were not antagonized by the classical anti-histamines. Almost two entire decades later the gene encoding the human H2R was cloned in 1991, even preceding the cloning of the human H1R by two years. Similar to the H1R the H2R is intronless. The H2R gene is located on chromosome 5q35.2 and encodes a 359 amino acid protein. So far only few single nucleotide polymorphisms have been identified in the H2R gene. These SNPs could not be associated with either atopic asthma or schizophrenia. The H2R is C-terminally palmitoylated and potentially N-glycosylated based on the presence of N-glycosylation sites in the N-terminal tail of the H2R. Whereas palmitoylation might be required for surface expression, the C-terminal tail is also important in H2R signalling, desensitization and internalization. The existence of putative H2R oligomers was proposed based on higher molecular weight species on Western blots. The human H2R is expressed ubiquitously, and can be found both in the CNS and peripheral tissue, including gastric parietal cells, vascular smooth muscle cells and neutrophils.

H2R mediated signalling
In contrast to the H1R, the H2R is primarily linked to activation of adenylyl cyclase via Gs-proteins. This Gs-coupling was established through observation of enhanced GTP-azidoanilide-labelling of Gs-like proteins in H2R expressing SF9 cells. The Gs-mediated increase in cAMP and subsequent activation of protein kinase A (PKA) can result in cell division, cell differentiation, ion transport and ion channel function. Besides being coupled to Gs-proteins, support for H2R
mediated $G_q$-signalling has emerged. The $H_2R$ has been suggested to activate c-fos, c-jun \(^71\), phospholipase C \(^{109,110}\) and increase Ca\(^{2+}\) concentrations \(^{109,111}\) as well as inhibit arachidonic acid release \(^{112}\). However, in CHO cells stably expressing the $H_2R$ no $G_q$-coupling could be identified \(^{88}\). Therefore, this putative $G_q/11$-coupling could be cell-type specific. Similar to the $H_1R$ the $H_2R$ can be activated in a ligand-independent manner \(^{113}\). This constitutive activity of the $H_2R$ has led to reclassification of many $H_2R$ antagonists to inverse agonists \(^{114}\).

**Specific ligands for the $H_2R$**

One of the few selective $H_2R$ agonists is amthamine (2-amino-5-(2-aminoethyl)-4-methylthiazole), which exhibits slight higher potency than histamine, but combined with reasonable selectivity. Recently, amthamine as well as other $H_2R$ agonists, including 4-methylhistamine, dimaprit and impromidine were shown to have higher affinity for the $H_2R$ than for the $H_2R$ \(^{115}\). Compared to $H_2R$ agonists many more selective $H_2R$ antagonists are available. Ligands like ranitidine, famotidine and tiotidine are potent antagonists/inverse agonists for the $H_2R$. The discovery of the $H_3R$ and $H_4R$ has revealed that some classical $H_2R$ antagonists such as burimamide are less selective as initially believed \(^{115}\).

**Physiological roles of the $H_2R$**

The $H_2R$ is involved in the release of gastric acid. Several $H_2R$ antagonists, cimetidine being the first, have been used successfully in treatment of gastric ulcers. Several of these therapeutic $H_2R$ antagonist have become blockbuster drug e.g. Zantac\textsuperscript{®} (ranitidine) and Pepcid\textsuperscript{®} (famotidine) \(^{116}\). However, the use of these $H_2R$ antagonist in treatment of peptic ulcers has been largely replaced by the more effective proton pump inhibitors, such as Losec\textsuperscript{®} (omeprazol) \(^{117}\). $H_2R$ are also involved in the regulation of cytokine and chemokine production and differentiation and maturation of a variety of cells in cardiac tissue, smooth muscle, and cells of the immune system \(^{118}\). Activation of the $H_2R$ can induce vascular and smooth muscle relaxation as well \(^{119}\). Elevated $H_1R$ and $H_2R$ mRNA levels have been detected in patients suffering from irritable bowel syndrome \(^{120}\). Together with the
H₄R, activation of the H₂R results in histamine-induced release of interleukin-16 from CD8⁺ T cells linking these receptors to play a role in asthma. The potential role for the H₂R in allergic asthma was further supported by the finding of H₂R mediated reductions in tumor necrosis factor α in rat peritoneal mast cells.

**Figure 1.9: Chemical structure of several H₂R ligands**

The histamine H₃ receptor

**Gene structure and expression**

In 1983 Arrang et al discovered a third member of the histamine receptor family. However, it would take sixteen more years before the gene encoding the human histamine H₃ receptor (H₃R) was cloned. The H₃R gene is located on chromosome 20q13.32-20q13.33, and unlike the previously cloned genes for the H₁- and H₂-receptors consists of at least two and perhaps three introns. The presence of intronic regions allows for the H₃R to be alternatively spliced. So far twenty different human H₃R isoforms have been identified. The majority of alternative spliced H₃R have an alternative length of intracellular loop 3.
general the H_{3(445)}R with a length of 445 amino acids is considered the full-length or wild-type H_{3}R. Besides alternative spliced H_{3}Rs, the existence of single nucleotide polymorphisms (SNPs) contributes to H_{3}R heterogeneity. At least three SNPs in the coding region of the H_{3}R are reported, the most important being the A280V H_{3}R, which might be linked to Shy-Drager syndrome. The H_{3}R shares low homology with the H_{1}R (22%) and H_{2}R (21.4%). This is in the same order as the homology between the H_{3}R and other members of the aminergic subfamily of GPCRs (20-27%).

The H_{3}R appears to be expressed almost exclusively in the central nervous system (CNS). However, H_{3}Rs can also be detected in other tissues. The H_{3}R is present both on histaminergic neurons (auto-receptors), as well as on non-histaminergic receptors (hetero-receptors). This means that activation of H_{3}R can inhibit the release of histamine as well as other neurotransmitters, such as dopamine, acetylcholine, serotonin, norepinephrine and \gamma-amino butyric acid.

**H_{3}R mediated signalling**

The involvement of G_{i/o}-proteins in H_{3}R signalling was initially demonstrated by inhibition of H_{3}R-mediated[^{35}S]GTP\gammaS binding with pertussis toxin. After the cloning of the H_{3}R these findings were encouraged through heterologous expression. Unlike the H_{1}- and H_{2}-receptors the H_{3}R couples to G_{i/o}-proteins, thus inhibiting the production of cAMP via adenyly cyclase. A decrease of cAMP results in a reduction in protein kinase A (PKA) activation and subsequent phosphorylation of cAMP responsive element binding proteins (CREB). As these CREB proteins can modulate gene transcription, H_{3}R activation has a negative influence on the expression of other proteins. H_{3}R activation has also been demonstrated to involve activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), phospholipase A\_2 (PLA\_2), inhibition of the Na\^+/H\^+ exchanger and lowering of intracellular Ca\^{2+} concentrations. Besides being activated by ligands the H_{3}R can also be constitutively activated. While this is not uncommon for GPCRs, the constitutive activity has been shown not only in vitro but also in vivo.
Specific ligands for the H\textsubscript{3}R

Most likely, due to the low homology of the H\textsubscript{3}R with the H\textsubscript{1}- and H\textsubscript{2}-receptors, most H\textsubscript{3}R agonists, including the classical H\textsubscript{3}R agonists R-\alpha-methylhistamine and N\textsuperscript{\alpha}-methylhistamine exhibit low affinity (∼10000 fold) for the H\textsubscript{1}R and the H\textsubscript{2}R. However, the recent discovery of the H\textsubscript{4}R made apparent that some of the more selective H\textsubscript{3}R agonists, such as immepip also share high affinity binding for the H\textsubscript{4}R.

Recently, successful efforts have been made to produce selective H\textsubscript{3}R agonists, including immethridine \cite{138} and the N-methylated immepip analogue methimmepip \cite{139}. The latter displays a 2000-fold selectivity over the H\textsubscript{4}R \cite{139}. Many potent and selective H\textsubscript{3}R antagonists have been developed. However, most imidazole-containing H\textsubscript{3}R antagonists, including thioperamide, proxyfan and GT2331 exhibit good affinity towards the H\textsubscript{4}R, as well. In order to design more selective H\textsubscript{3}R antagonists several non-imidazole-containing H\textsubscript{3}R antagonists have been developed. Among others, Abbott Laboratories (ABT-239 and A349821) and Johnson & Johnson pharmaceutics (JNJ 520785) have been successful in producing several potent selective H\textsubscript{3}R antagonists \cite{140-142}. Noteworthy are also the H\textsubscript{3}R inverse agonist clobenpropit, which behaves as partial agonist on the H\textsubscript{4}R \cite{115}, and proxyfan which is a so called protean agonist i.e. depending on the cell physiology proxyfan can exert H\textsubscript{3}R responses ranging from full agonist to full inverse agonist \cite{115}. 
Potential physiological roles of the H3R

The dual role of H3R as both hetero- and auto-receptor has led to the development of both antagonists as well as agonists as potential drugs. It has been suggested that inhibition of histamine release from histaminergic neurons, through activation of the H3R could induce sleep, and thus be used for treatment of insomnia \(^{143}\). Agonists of the H3R might be used for antinociceptive purposes \(^{144}\). H3R antagonists might be of therapeutical use in commercially interesting diseases. H3R antagonists have been demonstrated to be effective in reducing food intake of rodents \(^{145}\). As hetero-receptor the H3R is involved in modulation of neurotransmitter release. In particular the modulated release of acetylcholine makes the H3R an interesting target in treatment of neuropsychiatric diseases,
such as Alzheimer’s disease, Parkinson’s disease and attention-deficit hyperactivity disorder (ADHD).

**The histamine H₄ receptor**

Long before the cloning of the histamine H₄ receptor, evidence of a histamine receptor present in eosinophils was found. In 1975 Clark and co-workers found that a relatively low dose of histamine (in the range of 0.3-1.25 μM) caused migration of eosinophils. The effect of histamine could not be blocked by H₁- (mepyramine) or H₂- (metiamide) receptor antagonists. Histamine and Rα-methyl-histamine can induce calcium mobilization in eosinophils and the effect of these ligands could be blocked by thioperamide, burimamide and impromidine. However, the potency of Rα-methyl-histamine is lower than of histamine, which is inconsistent for H₃ mediated effects.

**H₄R gene structure**

With the clarification of the human genome, the search for other members of the histamine family by homology (BLAST) search was performed by several groups simultaneously leading to the discovery and cloning of a fourth histamine receptor. This H₄ receptor was located on chromosome 18q11.2. The gene for the histamine H₄ receptor spans over 20.6 kbp. Similar to the H₃ receptor, the H₄R gene consists of three exons and two introns. Transcription of the gene results in mRNA with a length of 3.7 kb. The open reading frame consists of 1173 bp or 390 amino acids. So far single nucleotide polymorphisms have been reported but either in the introns or 3’ untranslated region (3’ UTR). The 5’ flanking region contains several bindings motives (e.g. interferon stimulated response element; ISRE, nuclear factor κB; NF-κB, nuclear factor to interleukin 6; NF-IL6, interferon regulatory factor; IRF) for the binding of transcription factors. After the cloning of the human H₄R, genes corresponding to the H₄R were identified and cloned.
from mouse, rat, guinea pig, pig and monkey. The homology between the human and the rodent and pig H₄ are relatively low (65-72%). However, the monkey H₄R and human H₄R show higher homology (92%). This explains why the pharmacological profile of the human H₄Rs is similar to that of the monkey H₄R but is quite different from that of the rodent H₄R. ¹⁵⁵-¹⁵⁷

**Figure 1.11:** Location of the locus of the H₄R gene on chromosome 18

**Figure 1.12:** Genomic structure of the H₄R. Exon sequence in upper case and intron sequence in lower case. The sizes of exons are labeled by the numbers according to the coding sequence. The sizes of introns are estimated based on the genomic sequence. Figure adapted from ¹⁵¹

**H₄R protein structure**

The amino acid sequence, independently determined by the groups that cloned the H₄ receptor, is identical to each other with one exception. In the original cloning paper of Oda et al, three amino acids were reported, that were inconsistent with the sequence reported by the other groups namely the amino acids at position 138 (A₁₃₈V), 206 (H₂₀₆R), 253 (Q₂₅₃R). However, at the moment the sequence in NCBI that refers to sequence of Oda et al is the same as the sequence of the others, suggesting that the differences were most likely caused by sequencing errors. The H₄R protein contains two asparagines in the N-terminus (Asn⁵ and Asn⁹), which could function as possible glycosylation sites. Additionally the H₄R protein contains a disulphide bridge between the Cys⁸⁷ (extracellular loop 2) and Cys²⁶⁴ (EL 3) and a possible palmitoylation site in the C-terminus (Cys³⁷⁴). The receptor also contains several other hallmark features of (aminergic) G protein
coupled receptors: a conserved aspartates in TM2 and TM3, a DRY motif in TM3, conserved tryptophane residues in TM4 and TM6, a conserved proline in TM5 and a NPxxY motif in TM7. Using Western blot analysis Nguyen et al already showed hints of a unglycosylated (44 kDa), glycosylated (85 kDa) and putative oligomeric (>250 kDa) forms of the H₄ receptor.

In 2002 Shin and co-workers tried to identify the binding pocket of histamine to the H₄ receptor (Figure 1.13). They mutated several residues earlier found to be important for binding of histamine to the other members of histamine receptor family. They suggest that Asp⁹⁴ (3.32) and Glu¹⁸² are critical for histamine binding as mutations of either of these residues to alanine completely eliminated histamine binding. The Asp⁹⁴ might serve as counter-anion to the cationic amino group of histamine, whereas the Glu¹⁸² could interact with histamine by forming an ion-pair with the Nτ nitrogen atom. They also reported on the importance of residues Asn¹⁴⁷ (4.57) and Ser³²⁰ (6.20) in receptor activation.

Figure 1.13: A molecular model of the binding pocket of the H₄R. Asp⁹⁴ (3.32) in TM3 is expected to form an ion pair to the cationic amino group of histamine. Thr¹⁷⁸ (5.42) and Ser²⁷⁸ (5.43) in TM5 could form a hydrogen bond to the imidazole Nπ nitrogen. Glu¹⁸² (5.46) in TM5 could form an ion pair to the protonated imidazole Nτ nitrogen. Asn¹⁴⁷ (4.57) in TM4 and Ser³²⁰ (6.52) in TM6 point toward the central histamine-binding cavity. Figure adapted from.
The H₄ receptor shares highest homology with the histamine H₃ receptor; ~37% overall and ~58% for the transmembrane domains, whereas the homology with the H₁ and H₂ receptors is lower than 25% \(^{149}\). (Figure 1.14).

![Figure 1.14: Phylogenetic tree demonstrating homology between members of the aminergic receptors belonging to the superfamily A GPCRs. Figure adapted from \(^{159}\).](image)

**H₄R protein expression**

Northern blot and quantitative PCR analysis showed that the H₄ receptor is predominantly expressed in hematopoietic cells, particularly eosinophils, mast cells, basophils and T cells. Presence of the histamine H₄ receptor in the brain is contradictive and therefore still inconclusive. Zhu and co-workers found traces of H₄ receptor in *in situ* hybridization studies in mouse brain \(^{151}\), whereas also RNase protection assays performed on human brain tissue provided evidence for the existence for H₄ mRNA in brain \(^{148}\). However, Northern analysis performed by other groups reported the absence of H₄ receptor in the brain \(^{149,150,152}\). Since the transcription of the H₄ receptor gene is regulated by IL-10 and IL-13 \(^{150}\) it is possible that the H₄ receptor was absent under the conditions used for the
Northern blot assay. Although the H_4- and H_3- receptor apparently exhibit completely different expression patterns, they have been shown to be co-localized in nerve cells of human nasal mucosa.

**H_4R mediated signaling**

Similar to the H_3 receptor, stimulation of the H_4 receptor causes an inhibition in forskolin induced cAMP production\(^{148,151-153}\). This effect can be abolished by pre-treatment with pertussis toxin (PTX) suggesting that the receptor is coupled to the G_{i/o} G-proteins. The H_4 receptor has also been shown to activate mitogen activated protein kinase (MAPK) in HEK 293 cells upon histamine stimulation, but not in monocyte-derived dendritic cells\(^{161}\). The activation of MAPK in HEK293 is G_{i/o} mediated as the effect is blocked by PTX\(^{150}\). In transfected cells calcium mobilization could only be induced if the H_4 receptor was co-expressed with promiscuous (G_{a15} and G_{a16}) or chimeric G proteins, such as G_{aq15}, G_{aq1/2}, G_{aq3},\(^{148,150-152}\). Activation of the H_4R may also cause modulation of the cytokine induced JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) pathway in atopic and non-atopic lymphocytes\(^{162,163}\). Histamine activation of H_4R in non-atopic lymphocytes reduced the formation and phosphorylation of STAT1\(^{\alpha}\)\(^{162,163}\), whereas in atopic lymphocytes blockade of the H_4R with JNJ 7777120 resulted in an increase in STAT6 phosphorylation\(^{163}\). Constitutive activity was shown using a [\(^{35}\)S]GTP\(_{\gamma}\)S assay\(^{150}\) and a CRE-\(\beta\)-galactosidase reporter gene assay. In these assays thioperamide acts as inverse agonist\(^{148,150}\).

Histamine activation of the H_4 receptor was shown to induce chemotaxis and calcium mobilization from intracellular stores in murine mast cells. Both effects can be blocked by incubation with selective H_4 receptor antagonists (thioperamide and JNJ 7777120), PTX treatment and by inhibiting phospholipase C, using the PLC inhibitor U-73122\(^{164}\). Activation of the H_4 receptor leads to the release of G\(_{a}\) and G\(_{b\gamma}\) subunits. Most likely the G\(_{b\gamma}\) subunits activate phospholipase C, leading to production of DAG and InsP\(_3\). InsP\(_3\), possibly through interaction with an InsP\(_3\) receptor, releases calcium from intracellular storage vesicles (Figure 1.15).
Figure 1.15: Overview of H₄R mediated signaling pathways. The H₄R is a Go-α-coupled receptor inhibiting the production of cAMP via inhibition of adenylyl cyclase (AC). The reduction in cAMP will lead to a reduction in PKA activation and subsequent phosphorylation of the transcription factor CREB. The decrease PKA causes an increase in mitogen activated protein kinase (MAPK). The Gβγ subunit activates phospholipase C, which in turn hydrolyze phosphatidylinositol 4,5-biphosphate to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (InsP₃) releasing calcium from intracellular storage vesicles. Activation of H₄R has also causes inhibition of the cytokine induced JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) pathway. CR, chemokine receptor.

In human eosinophils, which do not express the H₃R, activation of the H₄R can induce shape changes. The effect was attributed to the H₄R since the H₄R agonist/H₃R antagonist clobenpropit as well as clozapine induced the cytoskeletal change. Additionally, histamine and clobenpropit were also able to induce actin polymerization. Histamine causes upregulation of the cell adhesion molecule CD11b/CD18 (Mac-1) and CD54 (ICAM-1). Upregulation of adhesion molecules on the cell surface is essential for cell spreading and the passage of (white) blood cells in the process of cell migration. In eosinophils histamine and clozapine were able to cause an increase in intracellular calcium. All these effects could be blocked by thioperamide. Histamine is able to induce chemotaxis of...
human eosinophils\textsuperscript{165,168}. However, earlier findings of Buckland et al., did not observe any significant histamine induced chemotaxis in eosinophils\textsuperscript{167}. Remarkably O’Reilly et al., showed histamine induced chemotaxis in eosinophils could be inhibited by thioperamide, but also by the H\textsubscript{4}R agonist clobenpropit\textsuperscript{168}. Nakayama et al found significant chemotaxis after treatment with CCL16/LEC, which could be blocked by thioperamide\textsuperscript{166}. Pretreatment of eosinophils with IL-5 led to a 4-fold increase in the chemotactic response of histamine\textsuperscript{168}. Preincubation with histamine has been reported to enhance the chemotactic potential of several chemokines including CCL7/MCP-3, CCL11/eotaxin and CCL24/eotaxin-2\textsuperscript{165,167}.

**Specific ligands for the H\textsubscript{4}R**

Reports on the cloning of the H\textsubscript{4}R also provided a limited characterization of the H\textsubscript{4}R pharmacology using several typical histamine receptor ligands. The relatively high homology with the H\textsubscript{3}R explains that most ligands that bind to the H\textsubscript{3}R also have affinity for the H\textsubscript{4}R. In most cases the affinity is lower for the H\textsubscript{4}R than for the H\textsubscript{3}R. Histamine appears equipotent on the H\textsubscript{4}R and H\textsubscript{3}R whereas N\textsuperscript{\textalpha}-methylhistamine and R(-)-\textalpha-methylhistamine are both >100 fold more potent at the H\textsubscript{3}R. Yet, immepip and imetit are only >10 fold more potent at the H\textsubscript{3}R. On the other hand clozapine and dimaprit exhibit higher affinity for the H\textsubscript{4} than for the H\textsubscript{3}R. Analysis of the binding data for several ligands on the H\textsubscript{4}R gave the following rank order: imetit>clobenpropit>histamine> immepip>thioperamide>NAMHA >RAMHA>dimaprit>clozapine\textsuperscript{148-153}. Thioperamide an inverse agonist on the H\textsubscript{3} receptor also acts as inverse agonist on the H\textsubscript{4}R. Clobenpropit is a remarkable ligand as it behaves as inverse agonist on the H\textsubscript{3}R, but as partial agonist on the H\textsubscript{4}R. Therefore, in several early studies on H\textsubscript{4}R functionality, clobenpropit was used to prove that an effect is H\textsubscript{4}R and not H\textsubscript{3}R mediated. However, in more recent years several potent and selective H\textsubscript{4}R ligands have been identified\textsuperscript{169,170} making the use of clobenpropit for this purpose obsolete.

So far few H\textsubscript{4}R selective ligands have been identified and it has been easier to produce H\textsubscript{3} specific ligands. In the search for more selective H\textsubscript{4} ligands, Hashimoto et al developed a series of chiral imidazole-containing ligands. The
most potent compound OUP-16 (Figure 1.16) is >40 fold more potent on the H₄R than on the H₃R. One of the first potent and selective H₄R antagonists was synthesized by Jablonowski et al in 2003. The compound JNJ 7777120 (Figure 1.16) showed >1000 fold selectivity over the other histamine receptors. Screening of benzimidazole analogs of JNJ 7777120 resulted in another selective H₄R antagonist, VUF6002 (Figure 1.16), with comparable affinity to JNJ 7777120. The same compound was also picked up in a screening by Johnson and Johnson (JNJ 10191584), as high affinity H₄R antagonist (pA₂ = 7.7), and showed no cross reactivity to a panel of over 50 receptors, transported and ion channels.

Figure 1.16: Chemical structure of several H₄R ligands
In search of potent selective agonists for the H₄R, a series of dibenzodiazepine derivatives was screened. Optimization of the tricyclic clozapine (Figure 1.16) lead to (E)-7-chloro-11-(4-methylpiperazin-1-yl)dibenzo[b,f][1,4]oxazepine. The ligand is a potent full agonist (pKi = 7.6, pEC₅₀ = 7.7), and is selective over the H₂R and H₃R (>300 fold), but not the H₁R. However, the screen provided useful information on the ligand binding pocket, which can be used in development of more potent and selective H₄R ligands. A remarkable finding came from the group of Yoshie, who reported that the chemokine CCL16/LEC was able to bind to and activate the H₄ receptor. CCL16 was shown to bind to the H₄ receptor with an affinity of 17 nM and induce calcium mobilization and chemotaxis of L1.2 cells.

Potential physiological roles of the H₄R

The exclusive expression pattern of the H₄R in cells belonging to the hematopoietic lineage, such as eosinophils, and mast cells, already early on led to speculation for this receptor to be play a role in immune, inflammatory diseases. The H₄R, histamine is involved in mast cell migration both in vitro and in vivo. Mast cell migration may play a role in allergic rhinitis and allergy. To study inflammation zymosan is often used to experimentally induce acute inflammation. In a peritonitis model in mice blockage of the H₄R with thioperamide or JNJ 7777120 showed a reduction in the accumulation of polymorphonuclear cells after zymosan injection, suggesting a role for the H₄R in general inflammation. In the same test model Takeshita et al also found that histamine induces leukotriene B₄ release. Leukotriene B₄ (LTB₄) is a potent chemotactic factor for neutrophils and has been postulated to play an important role in a variety of pathological conditions including rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease. By acting on the H₄R histamine stimulation was able to mimic the zymosan induced neutrophil release from murine bone marrow. The H₄R was also shown to play a role in the early phase inflammatory responses in carrageenan-induced acute inflammation. The H₄R is partly responsible for the release of interleukin-16 (IL-16) upon activation with histamine in CD⁸⁺ lymphocytes. IL-16 is a mediator found in lungs of asthma.
patients following challenge. Additionally, the H4R also causes the release of several other cytokines. For example, blockade of the H4R in T cells stimulated using dendritic cells (expressing H4R) led to a decrease of in IL-4, IL-6 and IL-7. Stimulation of H4R in monocyte-derived dendritic cells suppressed IL-12 production, potentially through activation of the transcription factor AP-1. IL-12 is a major Th1 driving cytokine. Therefore, a reduction of IL-12 may result in Th2-type immune responses, suggesting that the H4R may cause immune-modulatory effects. The H4R was further linked to allergic responses by the finding that H4R knock out mice, or mice treated with the H4R antagonist JNJ 7777120, showed decreased allergic lung inflammation. In these disease models decreases in both eosinophil and lymphocyte infiltration in the lung were observed. Based on the induction of scratching upon treatment of clobenpropit the H4R has been linked to be involved in itch in BalbC mice. Dunford et al, showed that histamine and 4-methylhistamine induced scratching in mice could be inhibited by treatment with the H4R antagonist JNJ 7777120 with higher potency then with H1R antagonists. This result suggests a potential role for H4R antagonists as therapeutic in chronic pruritic diseases that cannot be treated with H1R antagonists.

The H4R is abundantly present in synovial cells from patients suffering from rheumatoid arthritis. The amount of H4 receptor differed between patients, suggesting the possibility that there is a relation between the amount of H4 receptor and the duration and severity of rheumatoid arthritis. Moreover, a possible involvement of the H4R in ulcerative colitis has been demonstrated. Measurable effects of acute colitis induced by trinitrobenzene in rat, such as elevation of tumour necrosis factor-α (TNF-α), and histologically assessed increase in neutrophil infiltration, mucosal and submucosal thickness could be inhibited by the selective H4R antagonists JNJ 10191584 (VUF6002) and JNJ 7777120. In colon cancer cells (Caco-2 and COX-2-positive HT29) histamine was shown to increase COX-2 expression/activity, cell proliferation, and VEGF production. Treatment with either H2- or H4- receptor antagonists averted these effects, suggesting that these receptors may play a role in tumor progression e.g.
proliferation and angiogenesis. Recently Maslinska and co-workers reported the presence of H₄R protein on mammary epithelial cells of the human breast. Interestingly a strong presence of H₄R protein in fibroadenoma (benign tumour) and carcinoma tubulare (malignant tumour) was detected, suggesting a possible role for the H₄R in breast cancer.

In order to use H₄R antagonists in clinical trials they need to be evaluated for their pharmacokinetic and pharmacodynamic properties. The selective H₄ antagonist JNJ 7777120 has already been tested for its pharmacokinetic profile in rodent and dogs. The compound has a biological half life of 1-2.5 hours and a bioavailability after oral administration of 22% (rats) and 100% (dogs). In comparison JNJ 10191584 (VUF6002) the benzimidazole analogue of JNJ 7777120 had a slightly shorter half life of 1 hour.
1.3 Scope and aim of the thesis

In 2000 a fourth member of the histamine receptor family of GPCRs was discovered by several groups simultaneously\(^{148-153}\). Although this H\(_4\)R shares highest homology with the H\(_3\)R there is little overlap in tissue distribution. Whereas the H\(_3\)R is expressed mainly in the brain, the H\(_4\)R is expressed in hematopoietic cells such as, white blood cells, mast cells and dendritic cells.

At the time the Ph.D. project was initiated, still only little information was known about the H\(_4\)R. In order to obtain a better insight in the pharmacology of this novel receptor, a large set of H\(_1\)R, H\(_2\)R and H\(_3\)R ligands were screened on the H\(_4\)R. The results of this screen are summarized in chapter 2. In all living organisms DNA is transcribed to so-called messenger RNA and subsequently translated into proteins. In many cases the mRNA undergoes a special treatment in which specific regions (introns) are removed from the mRNA, and the remaining regions (exons) are fused together before translation. This process is known as splicing.

Whereas the genes encoding the H\(_1\)R and H\(_2\)R are intronless, both the genes encoding the H\(_3\)R and H\(_4\)R consist of several introns and exons. It is known that H\(_3\)R mRNA can be alternatively spliced giving rise to different H\(_3\)R isoforms. Some of these H\(_3\)R splice variants can play a dominant negative role on the wild-type H\(_3\)R. In chapter 4 the identification of and characterization of two splice variants of the H\(_4\)R is described. Additionally, the effect of these H\(_4\)R splice variants on the wild-type H\(_4\)R was investigated.

Unlike many other classes of proteins, GPCRs were thought for a long time to exist and function solely as monomeric entities. However, during the last decade of the previous century progressively data emerged suggesting that GPCRs may exist as higher oligomeric complexes. The study of GPCR oligomerization has grown exponentially the last years and has resulted in the publication of hundreds of articles on this topic. A large percentage of these papers concern GPCR belonging to the class of aminergic receptors, i.e. receptors that bind small endogenous biogenic amines, such as histamine, dopamine and adrenaline. In chapter 6, a review has been written summarizing the current state of research on oligomerization of aminergic receptors.
The study of oligomerization of the H₄R played an important part of the Ph.D. project. With the aim of studying homo- and hetero-oligomerization of the H₄R several biochemical and biophysical techniques were employed. The biophysical assays relied on the principal that one fluorescent conjugate can excite a second fluorescent moiety if in close proximity. The biochemical assays, benefited from the development of a specific H₄R antibody. In chapter 3 the results concerning homo-oligomerization of the H₄R and hetero-oligomerization between the H₄R and H₁R are described.

The H₄R is suggested to play a role in inflammation and is expressed in cells, which are known to also express several receptors belonging to the chemokine family. These chemokine receptors are acknowledged for their role in the immune system. Several viruses including several herpes viruses carry genes within their genome encoding GPCRs resembling chemokine receptors. The human cytomegalovirus is encoded by the chemokine receptor US28 and three other chemokine receptors. We found evidence that the H₄R may form hetero-oligomers with the US28 receptor. Chapter 6 describes the study of the interaction between the H₄R and US28 receptor and its potential functional implications.

An additional objective in this Ph.D. project has been to study the molecular pharmacology of the H₄R using biophysical approaches. The project focused on large scale production and purification of the H₄R using various strategies e.g. baculovirus and semliki forest virus expression systems, with the goal of using the purified receptor in analysis of the protein structure. A potentially effective strategy to obtain structural information on a protein is to produce large purified quantities of the protein of interest. The purified protein can subsequently be studied at high resolution by employing techniques such as X-ray crystallography, and Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS). Our efforts to produce large amounts of H₄R protein are described in chapter 7. We have employed several strategies to over-express the H₄R in different cell lines. These efforts include the use of viral overexpression systems, such as baculovirus and Semliki Forest virus as well as codon optimization of the H₄R.
CHAPTER 2

Evaluation of Histamine H₁-, H₂-, and H₃-Receptor Ligands at the Human Histamine H₄ Receptor: Identification of 4-Methylhistamine as the First Potent and Selective H₄ Receptor Agonist

Lim HD¹ and van Rijn RM¹, Ling P, Bakker RA, Thurmond RL, Leurs R., Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Johnson and Johnson Pharmaceuticals Research & Development, L.L.C., 3210 Merryfield Rd., San Diego CA 92121, California, USA

Adapted from Journal of Pharmacological and experimental therapeutics (2005), 4(3), 1310-21.

"Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved."
ABSTRACT

The histamine H₄ receptor (H₄R) is involved in the chemotaxis of leukocytes and mast cells to sites of inflammation and is suggested to be a potential drug target for asthma and allergy. So far, selective H₄R agonists have not been identified. In the present study we therefore evaluated the human H₄R (hH₄R) for its interaction with various known histaminergic ligands. Almost all of the tested H₁R and H₂R antagonists, including several important therapeutics, displaced less than 30% of specific [³H]histamine binding to the hH₄R at concentrations up to 10 μM. Most of the tested H₂R agonists and imidazole-based H₃R ligands show micromolar to nanomolar-range hH₄R affinity and these ligands exert different intrinsic hH₄R activities, ranging from full agonists to inverse agonists. Interestingly, we identified 4-methylhistamine as a high affinity H₄R ligand (Kᵢ = 50 nM) that has a >100-fold selectivity for the hH₄R over the other histamine receptor subtypes. Moreover, 4-methylhistamine potently activated the hH₄R (pEC₅₀ = 7.4 ± 0.1, α=1) and this response was competitively antagonized by the selective H₄R antagonist JNJ 7777120 (pA₂ = 7.8). The identification of 4-methylhistamine as a potent H₄R agonist is of major importance for future studies to unravel the physiological roles of the H₄R.

INTRODUCTION

Histamine exerts many (patho-)physiological effects through its interaction with four histamine receptor subtypes that all belong to the family of G-protein coupled receptors (Hough, 2001). The histamine H₁ receptor (H₁R) and H₂ receptor (H₂R) had been pharmacologically identified long before their cDNAs were cloned (Gantz et al., 1991, Yamashita et al., 1991) and have been successful blockbuster targets for more than two decades. The cDNA encoding the histamine H₃R was cloned more recently (Lovenberg et al., 1999), and bioinformatic analysis of human genome databases resulted in identification of the gene encoding the human H₄R (hH₄R) based on its sequence homology to the H₃R gene (37%) (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). While
4-methylhistamine, a potent and selective H₄R agonist

the hH₄R is mainly present in the nervous system, the hH₄R is distributed mainly in hematopoietic cells (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). The H₄R shows a different pharmacological profile compared to the closely related H₃R, although many H₃R ligands also interact with the H₄R. Like the H₃R, the H₄R couples to pertussis toxin-sensitive Gᵢₒ-proteins and thereby inhibits forskolin-induced cAMP production (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). In addition, the H₄R also activates MAP kinase (Morse et al., 2001) and mobilizes calcium in eosinophils and mast cells (Buckland et al., 2003; Hofstra et al., 2003).

The presence of the hH₄R on leukocytes and mast cells suggests that this new histamine receptor plays an important role in the modulation of the immune system. This hypothesis is supported by the fact that IL-10 and IL-13 modulate hH₄R expression (Morse et al., 2001) and that binding sites for cytokine-regulated transcription factors, like ISRE, IRF-1, NF-κB, and NF-IL6, are present upstream of the hH₄R gene (Coge et al., 2002). Physiological roles of the hH₄R include the control of IL-16 release by human CD8⁺ T cells (Gantner et al., 2002), chemotactic responses and cytoskeletal changes of human eosinophils (O’Reilly et al., 2002; Buckland et al., 2003; Ling et al., 2004), chemotaxis and intracellular calcium mobilization in mast cells (Hofstra et al., 2003), and control of leukotriene B₄ production by mast cells that subsequently lead to neutrophil recruitment into peritoneum (Takeshita et al., 2003; Thurmond et al., 2004). These studies suggest that the hH₄R is a potential drug target for immune system-related diseases.

Until recently, potent and selective H₄R ligands were not available. In the early studies, the H₃R antagonist thioperamide was identified as an equally effective H₄R antagonist (Oda et al., 2001). High-throughput screening and subsequent medicinal chemistry efforts recently identified the indolylpiperazine JNJ 7777120 (Jablonski et al., 2003; Thurmond et al., 2004) and the related benzimidazole analog VUF 6002 (Terzioglu et al., 2004) as selective and potent H₄R antagonists. Studies directed towards selective H₄R agonists have so far been less successful. Burimamide, clozapine, and clobenpropit are all known to act as H₄R agonists, and
clozapine and clobenpropit have been proven useful for initial pharmacological studies (Gantner et al., 2002; Buckland et al., 2003; Bell et al., 2004; Ling et al., 2004). However, their non-selectiveness for the H₄R limits their use as H₄R agonists.

In our search for selective H₄R agonists, many known histaminergic ligands of different structural classes, including several important therapeutics, were evaluated for their interaction with the hH₄R. Our studies resulted in the identification of 4-methylhistamine, a presumed moderately active and selective H₂R agonist (Durant et al., 1975), as a high affinity H₄R agonist with a more than 100-fold selectivity over the H₁R, H₂R, and H₃R.

RESULTS

**Pharmacological characteristion of the hH₄R expressed in SK-N-MC cells**

Stable transfection of the human H₄R (hH₄R) cDNA in SK-N-MC cells resulted in the expression of functional hH₄R proteins. The hH₄R could be labeled with both agonist and antagonist radioligands. The H₄R agonist [³H]histamine shows saturable binding to the expressed H₄R with a minimal amount of non-specific binding (Figure 2.1A). Analysis of the [³H]histamine saturation binding yielded a Kᵣ value of 11 ± 1.0 nM (n=6) and a Bₘᵢᵦ value of 1.8 ± 0.4 pmol/mg protein. Recently, JNJ 7777120 was described as a selective H₄R antagonist (Jablonowski et al., 2003). In our hands, the non-imidazole JNJ 7777120 shows a 300-fold selectivity for the hH₄R (pKᵦ = 7.8 ± 0.1 against [³H]histamine) over the hH₃R (pKᵦ = 5.3 ± 0.1 against [³H]Nα-methylhistamine), allowing the use of [³H]JNJ 7777120 to label the H₄R (Thurmond et al., 2004). The H₄R antagonist [³H]JNJ 7777120 exhibits a somewhat higher level of non-specific binding to hH₄R expressing SK-N-MC cells, but also binds saturaibly and shows an equipotent affinity (Kᵦ = 11 ± 3.6 nM, n=3) and results in a Bₘᵢᵦ value of 1.7 ± 0.4 pmol /mg protein (Figure 2.1B). The binding of either 10 nM [³H]histamine (Figure 2.1C) or 10 nM [³H]JNJ 7777120 (data not shown) to the hH₄R is fully displaced by histamine (pKᵦ = 7.8 ± 0.1), the H₃/₄R antagonist thioperamide (pKᵦ = 6.9 ± 0.1) and the H₄R agonist/H₃R antagonist
clobenpropit (pKᵢ = 8.1 ± 0.1), in a good agreement with the results reported previously (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001).

The SK-N-MC/hH₄ cells used in this study co-express a CRE-controlled β-galactosidase reporter gene and can therefore also be used for a functional analysis of H₄R ligands. Stimulation of the hH₄R with histamine resulted in the inhibition (58±3%, n = 16) of the forskolin-stimulated (1 µM) cAMP-mediated reporter gene transcription with a pEC₅₀ value of 7.7 ± 0.1 (n = 16) (Figure 2.1D). Treatment of SK-N-MC/hH₄ cells with the Gαᵢ/o protein inhibitor pertussis toxin (100ng/ml for 16 hours) completely inhibited histamine induced responses, confirming the coupling of the H₄R to Gαᵢ/o proteins (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). In our hands, histamine exerted the maximally observed level of inhibition in this assay, and is therefore referred to as a full agonist (intrinsic activity α=1). As reported before (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001), clobenpropit acts as a potent partial H₄R agonist with a pEC₅₀ value of 7.7 ± 0.1 (n = 3) and an intrinsic activity of 0.8 (Figure 2.1D).

Treatment of SK-N-MC/hH₄ cells with pertussis toxin (100ng/ml for 16 hours) resulted in an increase of 1 µM forskolin-stimulated CRE activity by 130 ± 3%, suggesting that the H₄R shows a detectable level of constitutive activity in the SK-N-MC/hH₄ cells. In line with earlier observations on inverse agonism by thioperamide (Liu et al., 2001a; Morse et al., 2001), 1 µM forskolin-stimulated CRE activity was increased by thioperamide with a pEC₅₀ value of 7.0 ± 0.1 (Figure 2.1D). The inhibition of the constitutive activity of the H₄R by thioperamide was of the same magnitude as observed after treatment with pertussis toxin and thioperamide is referred to as a full inverse H₄R agonist (intrinsic activity α=-1).
The cell line SK-N-MC/hH4 stably expresses functional hH4R and CRE-control β-galactosidase. The homogenate of SK-N-MC/hH4 shows a saturable binding to H4R agonist [3H]histamine (A) with a minimal amount of nonspecific binding and also to H4R antagonist [3H]JNJ 7777120 (B) with a higher level of nonspecific binding. The binding of [3H]histamine to the hH4R is nicely inhibited by H3/4R ligands, displaying a one-binding binding site property (C). The full agonist histamine and partial agonist clobenpropit hH4R inhibits the 1 μM forskolin-induced cAMP formation, which is manifested as β-galactosidase activity, while the inverse agonist thioperamide dose-dependently blocks the hH4R constitutive activity (D).

In SK-N-MC/hH4 cells the cAMP-driven β-galactosidase reporter-gene transcription can also be activated by endogenously expressed Gαs protein coupled β adrenergic receptors (Bahouth et al., 2001). The β2 adrenergic receptor agonist feneterol induced β-galactosidase activity to a similar extend to that of forskolin with a pEC50 value of 6.9 ± 0.1 (n = 6). H4R activation by histamine inhibited the 100 nM feneterol-induced β-galactosidase activity for 39 ± 3 % with a pEC50 value of 7.4 ± 0.1 (n = 7). However, inverse agonistic activity of thioperamide, at the hH4R, could not be easily demonstrated with a feneterol-based assay system (data...
4-methylhistamine, a potent and selective \( H_4 \)R agonist

not shown). The evaluation of the functional activity of all the various histaminergic ligands was therefore performed using forskolin (1 µM) stimulated SK-N-MC/hH\(_4\) cells.

All compounds were preliminarily tested as displacers of \(^3\text{H}\)histamine binding to the hH\(_4\)R expressed in SK-N-MC/hH\(_4\) cells at a concentration of 10 \( \mu \text{M} \). Compounds inhibiting the specific binding of 10 nM \(^3\text{H}\)histamine to the hH\(_4\)R by \( \leq 30\% \) are expected to have a \( K_i > 10 \mu \text{M} \) (based on the Cheng and Prusoff equation \( K_i = IC_{50}/(1+([\text{radioligand}]/K_d)) \)), and were excluded for further testing. Active compounds (displacement \( > 30\% \)) were tested more extensively in both \(^3\text{H}\)histamine displacement studies and the CRE-\( \beta \)-galactosidase based functional H\(_4\)R assay.

**Most H\(_1\)R ligands are devoid of H\(_4\)R activity**

Histamine potently displaces \(^3\text{H}\)histamine from the hH\(_4\)R with a \( pK_i \) value of 7.8 ± 0.1 (Table 2.1), while H\(_1\)R agonists with substituents at the 2 position of the imidazole ring show significantly lower affinities. Substitution of the imidazole ring with either a small methyl or large 3-bromophenyl substituent is not tolerated and causes an almost 100-fold drop of affinity. Bulkier substituents at the 2-position (1,1-diphenylpropyl in histaprodifen) even result in a total loss of affinity for the hH\(_4\)R (Table 2.1). Agonists, lacking the imidazole ring, like 2-(2-thiazolyl)ethylamine (TEA), 2-pyridylethylamine (PEA) or 8R-lisuride (Bakker 2004), are also not active at the hH\(_4\)R (Table 2.1).

Following an initial report that the H\(_4\)R can be labeled with \(^3\text{H}\)-mepyramine (Nguyen et al., 2001), a large number of H\(_1\)R antagonists (Table 2.1), including many clinically relevant drugs, were evaluated for their hH\(_4\)R affinity as well. Almost all tested H\(_1\)R antagonists, including mepyramine, showed \( pK_i \) values <5 (Table 2.1) and did not show functional activity at 1 and 10 \( \mu \text{M} \) at the hH\(_4\)R (data not shown). Although structurally similar to some tricyclic H\(_1\)R antagonists devoid of H\(_4\)R affinity, clozapine binds with moderate potency to the hH\(_4\)R (\( pK_i 6.7 \pm 0.1 \)) and exerts full agonistic activity at the hH\(_4\)R with a pEC\(_{50}\) value of 6.8 ± 0.1 (n = 5). N-desmethyl clozapine, a clozapine metabolite, showed a slightly decreased affinity
(pKᵢ 6.5 ± 0.1), while N-oxide clozapine, another clozapine metabolite, is totally devoid of hH₄R affinity. Further, we evaluated clozapine analogs of therapeutic importance as well. Loxapine and amoxapine showed >10-fold lower affinity (pKᵢ 5.4 ± 0.1 and 5.3 ± 0.1, respectively), while octoclothepin did not show binding for the hH₄R at all.

**Table 2.1.** Activity of H₁R ligands at the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least 3 independent experiments.

<table>
<thead>
<tr>
<th>H₁R ligand⁵</th>
<th>H₁R activity</th>
<th>pKi⁴</th>
<th>pEC₅₀ b</th>
<th>α c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>agonist</td>
<td>7.8 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>2-methylhistamine</td>
<td>agonist</td>
<td>6.1 ± 0.1</td>
<td>5.9 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>2-(3-bromophenyl)histamine</td>
<td>agonist</td>
<td>6.0 ± 0.1</td>
<td>5.9 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Histaprodifen</td>
<td>agonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>2-(2-thiazolyl)ethylamine (TEA)</td>
<td>agonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>2-Pyridylethylamine (PEA)</td>
<td>agonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>8R-lisuride</td>
<td>agonist</td>
<td>5.3 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Azatidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clozapine</td>
<td>antagonist</td>
<td>6.7 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>Amoxapine</td>
<td>antagonist</td>
<td>5.3 ±0.1</td>
<td>6.7 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Loxapine</td>
<td>antagonist</td>
<td>5.4 ±0.1</td>
<td>6.2 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Octoclothepin</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ebastine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hydroxyzine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Loratidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mizolastine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mepyramine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>R(+)terfenadine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

⁴ pKi values were determined with [³H]histamine displacement assay.

⁵ pEC₅₀ values show the inhibition of 1 μM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₃ or SK-N-MC/hH₄ cells.

⁶ α is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for inverse agonistic activity.

⁵ The H₁R antagonists Chlorpheniramine, Cyproheptadine, Desipramine, Dexchlorpheniramine, Diphenhydramine, Doxepine, Imipramine, Ketotifen, Mianserine, Octoclothepin, ORG-3770, Promethazine, S(-)-terfenadine, Tripelennamine, and Triprolidine also have pKi values < 5.

⁶ due to non H₁R-mediated effects of 8R-lisuride, the pEC₅₀ value was not determined.

n.d. = not determined.
Some H2R ligands act as hH4R agonists

Within the series of known H2R agonists that we have tested, only some ligands retain H4R activity. Replacement of the imidazol ring of histamine in the selective H2R agonists amthamine and amselamine (Leurs et al., 1994) results in a total loss of hH4R activity at concentrations up to 10 μM. Dimaprit, a H2R agonist/H3R antagonist lacking an imidazole group, binds the H4R with moderate affinity, showing a pKi value of 6.5 ± 0.1, and exerts partial H4R agonistic activity (Table 2.2). Impromidine, which was reported to bind to both H2R and H3R, also binds potently to the hH4R with a pKi value of 7.6 ± 0.1 and acts as a partial H4R agonist (α = 0.5). Both the S and R enantiomers of the related sopromidine bind respectively >10 and >100 times less potently. In fact, the first reported H2R selective agonist 4-methylhistamine (Durant et al., 1975) is the only known H2R agonist that also acts as full agonist at the H4R (Table 2.2). 4-Methylhistamine binds two times less potent than histamine to the hH4R, exhibiting a pKi value of 7.3 ± 0.1 (n = 3).

Most tested H2R antagonists, including cimetidine, mifentidine, aminopotentidine, ranitidine, famotidine, and tiotidine, only displaced < 30% of 10 nM [3H]histamine binding to the hH4R. Only the H2/3R ligand burimamide shows a good affinity for the hH4R pKi = 7.4 ± 0.1 (Table 2.2). Moreover, burimamide acted as a potent, albeit partial H4R agonist (pEC50 = 7.7 ± 0.1, α = 0.7). Previously, we reported on various burimamide analogs as H3R antagonists. In our search for H4R selective ligands various burimamide analogs were therefore investigated for their H4R activity.
Table 2.2. Activity of H₄R ligands at the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least 3 independent experiments

<table>
<thead>
<tr>
<th>H₄R ligand</th>
<th>H₄R activity</th>
<th>pKiᵃ</th>
<th>pEC₅₀ᵇ</th>
<th>αᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylhistamine</td>
<td>agonist</td>
<td>7.3 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>Anthamine</td>
<td>agonist</td>
<td>5.3 ± 0.1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>agonist</td>
<td>5.6 ± 0.1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Dimaprit</td>
<td>agonist</td>
<td>6.5 ± 0.1</td>
<td>5.8 ± 0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Imipromidine</td>
<td>agonist</td>
<td>7.6 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>S(+) sopromidine</td>
<td>agonist</td>
<td>5.5 ± 0.1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>R(-) sopromidine</td>
<td>agonist</td>
<td>6.1 ± 0.1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Aminopotentidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Burimamide</td>
<td>antagonist</td>
<td>7.4 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Famotidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mifenidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tiotidine</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

ᵃ pKi values were determined with [³H]histamine displacement assay.
ᵇ pEC₅₀ values show the inhibition of 1 μM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₃ or SK-N-MC/hH₄ cells.
ᶜ α is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for inverse agonistic activity.

Due to non H₄R-mediated effects of 8R-lisuride, the pEC₅₀ value was not determined.

In this series of compounds, the presence of an isopropyl (VUF 4683 and VUF 4616) or cyclohexyl (VUF 4617) moiety adjacent to the thiourea group is optimal for hH₄R affinity (Table 2.3). Interestingly, this series of closely related compounds exerts partial agonistic, neutral antagonistic, and inverse agonistic activities at the hH₄R (Table 2.3, Figure 2.2A). Substitution of the thiourea with aromatic substituents, like a benzyl group in VUF 4686, results in a loss of H₄R agonistic activity. A total loss of agonistic H₄R activity, but not affinity (pKi = 7.6 ± 0.1) is surprisingly observed for VUF 4614. As can be seen in Figure 2.2B, VUF 4614 was able to competitively block the H₄ agonistic responses of histamine (Figure 2.2B), resulting in a pA₂ value of 6.8. Finally, within this series we identified VUF 4742 as...
4-methylhistamine, a potent and selective H₄R agonist

an hH₄R inverse agonist (Figure 2.2). This burimamide analog bound with moderate affinity to the H₄R (pKᵢ = 6.9 ± 0.1, n = 4) and acted as a full inverse agonist with a pEC₅₀ value of 7.2 ± 0.1 (n =5), in accordance with its binding affinity.

Table 2.3. Activity of burimamide analogs at the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least 3 independent experiments.

<table>
<thead>
<tr>
<th>ligand</th>
<th>n</th>
<th>R</th>
<th>pKᵢᵇ</th>
<th>pEC₅₀ᵇ</th>
<th>αᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>burimamide</td>
<td>4</td>
<td>methyl</td>
<td>7.4 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>VUF 4681</td>
<td>4</td>
<td>ethyl</td>
<td>7.6 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>VUF 4682</td>
<td>4</td>
<td>n-propyl</td>
<td>8.0 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>VUF 4683</td>
<td>4</td>
<td>isopropyl</td>
<td>8.1 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>VUF 4686</td>
<td>4</td>
<td>benzyl</td>
<td>7.3 ± 0.1</td>
<td>7.1 ± 0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>VUF 4687</td>
<td>4</td>
<td>ethylbenzyl</td>
<td>7.2 ± 0.1</td>
<td>5.9 ± 0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>VUF 4614</td>
<td>5</td>
<td>ethyl</td>
<td>7.6 ± 0.1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>VUF 4616</td>
<td>5</td>
<td>isopropyl</td>
<td>7.9 ± 0.1</td>
<td>d</td>
<td>n.d.</td>
</tr>
<tr>
<td>VUF 4617</td>
<td>5</td>
<td>cyclohexyl</td>
<td>7.9 ± 0.1</td>
<td>d</td>
<td>n.d.</td>
</tr>
<tr>
<td>VUF 4742</td>
<td>5</td>
<td>4-chlorobenzyl</td>
<td>6.9 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>-1</td>
</tr>
</tbody>
</table>

ᵃ pKᵢ values were determined with [³H]histamine displacement assay.
ᵇ pEC₅₀ values show the inhibition of 1 μM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₃ or SK-N-MC/hH₄ cells.
ᶜ α is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for inverse agonistic activity.
ᵈ These compounds exerted a biphasic response: a slight decrease of forskolin stimulated β-galactosidase activity at concentration up to 1 μM and an increase at concentration ≥ 10 μM. The latter effect is not mediated by the hH₄R, as it cannot be blocked by H₄R antagonist JNJ 7777120.

n.d. = not determined.
Figure 2.2. Burimamide analogs exert different intrinsic activities at the hH4R. (A) Burimamide acts as a H4R partial agonist, which dose dependently inhibits 1 μM forskolin-induced cAMP in SK-N-MC/hH4 cells, which is manifested as reporter β-galactosidase activity. Modifications of burimamide structure (Table 2.2) result in changes of ligand intrinsic activity, ranging from agonistic (VUF 4683, burimamide, VUF 4686) to neutral antagonist (VUF 4614) and inverse agonist (VUF 4742). (B) The neutral antagonist VUF 4614 competitively antagonizes histamine activity at the hH4R, resulting in a rightward shift of histamine dose-response curve.

Evaluation of H3R ligands at the hH4R

In view of the high sequence homology between the H3R and the H4R, it is not surprising that in the initial studies some H3R ligands were identified as H4R ligands as well. We therefore characterized in this study a large set of known H3R ligands for their interaction with the H4R. The histamine analogs Nα-methylhistamine, (R)-α-methylhistamine, and (S)-α-methylhistamine show an almost 2 order lower affinity for the hH4R than for the hH3R. However, the hH4R retains some level of stereo-selectivity for (R)-α- (pKi = 6.6 ± 0.1) and (S)-α-methylhistamine (pKi = 5.4 ± 0.1) (Table 2.4). Increasing the spacer-length between imidazole and amine group from 2 carbon atoms (histamine, pKi = 7.8 ± 0.1) to 3 carbon atoms (homohistamine, pKi = 7.5 ± 0.1) slightly decreases the affinity for the hH4R, while 4 carbon atoms (imbutamine, pKi = 8.0 ± 0.1) results in a slightly higher hH4R affinity. A further increase of the spacer length proved to be detrimental for hH4R affinity. The highly potent H3R agonist impentamine shows only moderate affinity at the hH4R (pKi = 6.6 ± 0.1) (Table 2.4). Interestingly, besides the affinity, impentamine also loses intrinsic activity for the hH4R (α = 0). Previously identified
4-methylhistamine, a potent and selective H₄R agonist

H₃R agonists, including immeip, imetit, and VUF 8328 (an imetit analog) (Wieland et al., 2001), also potently bind the H₄R with pKᵢ values of 7.7 ± 0.1, 8.2 ± 0.1, and 8.0 ± 0.1, respectively. At the H₄R these ligands also act as agonists, but exert somewhat lower intrinsic activity (α values of 0.9, 0.9, and 0.6, respectively) (Table 2.4).

Table 2.4. Activity of H₄R ligands at the hH₃R and the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least 3 independent experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>hH₃R</th>
<th>hH₄R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢᵃ</td>
<td>pEC₅₀ᵇ</td>
</tr>
<tr>
<td>Histamine</td>
<td>8.0 ± 0.1</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>N²-methylhistamine</td>
<td>8.4 ± 0.1</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>(R)-α-methylhistamine</td>
<td>8.2 ± 0.1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>(S)-α-methylhistamine</td>
<td>7.2 ± 0.1</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>Homohistamine</td>
<td>7.3 ± 0.1</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Imbutamine</td>
<td>8.4 ± 0.1</td>
<td>9.2 ± 0.1</td>
</tr>
<tr>
<td>Impentamine</td>
<td>8.3 ± 0.1</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>Immepip</td>
<td>9.3 ± 0.1</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td>Methimmepip</td>
<td>9.0 ± 0.1</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td>Immethridine</td>
<td>9.1 ± 0.1</td>
<td>9.8 ± 0.1</td>
</tr>
<tr>
<td>Imetit</td>
<td>8.8 ± 0.1</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td>VUF 8328</td>
<td>8.5 ± 0.1</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>8.6 ± 0.1</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>Iodophenpropit</td>
<td>8.2 ± 0.1</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>Impromidine</td>
<td>6.8 ± 0.1</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>Burimamide</td>
<td>7.9 ± 0.1</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>7.3 ± 0.1</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>Proxyfan</td>
<td>7.9 ± 0.1</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>Iodoproxyfan</td>
<td>9.2 ± 0.1</td>
<td>10.3 ± 0.1</td>
</tr>
<tr>
<td>JNJ 7777120</td>
<td>5.3 ± 0.1</td>
<td>6.0 ± 0.1</td>
</tr>
</tbody>
</table>

ᵃpKᵢ values were determined with [³H]histamine displacement assay.
ᵇpKᵢ values were determined with [³H]N²-methylhistamine displacement assay.
ᶜpEC₅₀ values show the inhibition of 1 μM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₃ or SK-N-MC/hH₄ cells.
ᵈα is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for inverse agonistic activity.
As reported previously (Kitbunnadaj et al., 2004), the recently identified H3R agonist immethridine (pKi = 9.1 ± 0.1) binds much less potently to the hH4R (pKi = 6.6 ± 0.1) and is also not able to fully activate the H4R (Table 2.4).

Figure 2.3. Iodophenpropit is an hH4R neutral antagonist. (A) SK-N-MC/hH4 cells, which stably express functional hH4R and CRE-control β-galactosidase, were treated with 1 μM forskolin in the presence of various concentrations of histamine, thioperamide, clobenpropit, and iodophenpropit for 6 hours, whereafter β-galactosidase activity was determined spectrophotometrically at 420 nm. Histamine and clobenpropit dose-dependently inhibit forskolin-induced β-galactosidase (agonistic activity) at the hH4R, while thioperamide causes increase of β-galactosidase, indicating a blockade of hH4R constitutive activity. Iodophenpropit does not change forskolin-induced β-galactosidase activity although it binds to the hH4R, indicating it is a neutral antagonist. (B) While clobenpropit is a partial agonist and iodophenpropit is a neutral antagonist at the H4R, they are both inverse agonists at the hH3R. (C) Iodophenpropit (IPP) antagonizes activity of histamine at the hH4R, resulting in a rightward shift of histamine dose-response curve. (D) Schild plot analysis of iodophenpropit antagonism against histamine results in a pA2 value of 8.0.

In agreement with our findings with Nα-methylhistamine, the methylated immepip analog methimepip show a large selectivity for the hH3R (pKi = 9.0 ± 0.1) over the
4-methylhistamine, a potent and selective H₄R agonist

hH₄R (pKᵢ = 5.7 ± 0.1), as reported previously (Kitbunnadaj et al., 2005). Also various H₃R antagonists bind to the hH₄R (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). In our hands the isothiourea-based H₃R antagonists clobenpropit and iodophenpropit both potently bind to the hH₄R (Table 2.4). Yet, both ligands have very distinguishable intrinsic activities at the H₄R. Clobenpropit, which acts as inverse agonist at the hH₃R (Wieland et al., 2001) behaves as a potent partial agonist at the hH₄R (Table 2.4 and Figure 2.3A and B). In contrast, iodophenpropit, which also acts as an inverse agonist at the hH₃R (Wieland et al., 2001) behaves as a neutral antagonist at the hH₄R with a pKᵢ value of 7.9 ± 0.1 (n = 6) (Table 2.4 and Figure 2.3A and B). As expected, iodophenpropit competitively antagonized the action of histamine at the hH₄R, yielding a linear Schild-plot and a pA₂ value of 8.0 (Figure 2.3C and D), in accordance with its binding affinity. Besides clobenpropit, also the known H₃R ligands proxyfan and the related iodoproxyfan show reasonable H₄R affinity with pKᵢ values of 7.3 ± 0.1 and 7.9 ± 0.1 respectively. As observed for their action at the H₃R, both compounds act as partial agonist at the H₄R (Table 2.4).

Evaluation of the potential use of [¹²⁵I]iodophenpropit as H₄R radioligand

As reported in the previous section and in other studies, the H₄R can be labeled with either [³H]histamine or the H₄R antagonist [³H]JNJ 7777120 (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001; Thurmond et al., 2004). Previously, we described [¹²⁵I]iodophenpropit as a suitable high affinity H₃R radioligand (Jansen et al., 1992). Considering, the relatively high affinity of iodophenpropit at the hH₄R and its high sensitivity, we investigated the potential of this radioligand to label the H₄R. Due to the H₄R affinity of [¹²⁵I]iodophenpropit saturation binding experiments were not feasible. We therefore used homologous [¹²⁵I]iodophenpropit displacement analysis to determine a Kᵢ value of 34.4 ± 4.1 nM for [¹²⁵I]iodophenpropit. The Bₘₐₓ value obtained using homologous [¹²⁵I]iodophenpropit-binding displacement experiments (3.8 ± 0.4 pmol/mg protein) is approximately 2 times higher than those obtained with either [³H]histamine (1.8 ± 0.4 pmol/mg protein) and [³H]JNJ 7777120 (1.7 ± 0.4 pmol/mg protein). [¹²⁵I]Iodophenpropit binding to membranes of SK-N-MC/hH₄ cells was
nicely displaced by a variety of H3 ligands (Figure 2.4), despite a high level of non-specific binding of approximately 60% as determined with 10 μM imetit. However, iodophenpropit and the related clobenpropit also displaced the non-specific binding, resulting in a multiple site binding profile. The pKi values of compounds for the hH4R obtained using [125I]iodophenpropit displacement studies correlate well with their corresponding values obtained using displacement of either [3H]histamine or [3H]JNJ 7777120 binding to the hH4R; only the pKi value of thioperamide obtained using [125I]iodophenpropit displacement studies appears to deviate somewhat from the pKi values obtained using either [3H]histamine or [3H]JNJ 7777120 displacement studies (Figure 2.4 and Table 2.5).

Table 2.5. hH4R affinity for selected H4R ligands as determined with displacement of the binding of [3H]histamine, [3H]JNJ 7777120 or [125I]iodophenpropit. Data shown are mean ± standard error of mean of at least 3 independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKi [3H]histamine</th>
<th>pKi [3H]JNJ 7777120</th>
<th>pKi [125I]iodophenpropit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>7.8 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>4-Methylhistamine</td>
<td>7.3 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>not determined</td>
</tr>
<tr>
<td>Immepip</td>
<td>7.7 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Clozapine</td>
<td>6.7 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>8.1 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Iodophenpropit</td>
<td>7.9 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>6.9 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>JNJ 7777120</td>
<td>7.8 ± 0.1</td>
<td>7.8 ± 0.2</td>
<td>not determined</td>
</tr>
</tbody>
</table>

Kd (nM)  
20.9 ± 1.6  
11.1 ± 3.6  
34.4 ± 4.1

Bmax (pmol/mg protein)  
1.8 ± 0.4  
1.7 ± 0.4  
3.8 ± 0.4

* Determined by homologous displacement analysis, employing equation $K_d = IC_{50} - [radio-ligand]$. 

Table 2.5. hH4R affinity for selected H4R ligands as determined with displacement of the binding of [3H]histamine, [3H]JNJ 7777120 or [125I]iodophenpropit. Data shown are mean ± standard error of mean of at least 3 independent experiments.
4-methylhistamine, a potent and selective H4R agonist

Figure 2.4. Analysis of ligand binding to the hH4R using [125I]iodophenpropit. (A) Homogenate of SK-N-MC/hH4 cells were incubated with 0.1 nM [125I]iodophenpropit in the presence of different concentrations of H3/4 ligands and the nonspecific activity was determined with 1 μM imetit. The data, shown as % of specific binding, fit nicely into a one-site ligand-receptor model.

4-methylhistamine as a selective H4R agonist

Following our initial observation of the relative high affinity of 4-methylhistamine for the hH4R, this histamine analog was evaluated in more detail. 4-Methylhistamine does not only have high affinity for the hH4R (pKi = 7.3 ± 0.1, n = 3), but it also exhibits considerable selectivity for the hH4R over the other three human histamine receptors (Figure 2.5A). The human histamine H1, H2 and H3 receptors were tested for their interaction with 4-methylhistamine, using respectively 1 nM [3H]mepyramine (Kd = 1.6 nM), 0.5 nM [125I]iodoaminopotentidine (Kd = 0.5 nM) and 1 nM [3H]Nα-methylhistamine (Kd = 2.9 nM) binding to homogenates of transfected cells. As can be seen in Figure 2.5A, 4-methylhistamine shows highest affinity for the hH4R and binds considerably less potently to the other histamine receptors, resulting in a >100-fold and >100,000-fold selectivity over the H3R and H2R, and H1R, respectively. 4-Methyl histamine does not only bind to the hH4R, but also has a high affinity, albeit reduced compared to the hH4R, for the mouse and rat H4R with Kd values of 73 and 55 nM, respectively (Figure 2.5B). Moreover, 4-methylhistamine exerts full agonistic activity at the hH4R, resulting in a pEC50 value of 7.4 ± 0.1 (α = 1, n = 5). In contrast, 4-methylhistamine exhibits only moderate affinity for the hH3R (pKi = 5.2 ± 0.1, n = 4) and partial agonistic hH3R activity.
The hH₄R agonistic effects of 4-methylhistamine can be antagonized by the selective H₄R antagonist JNJ 7777120 (Figure 2.5D). Schild-plot analysis of the JNJ 7777120 antagonism of the 4-methylhistamine-induced hH₄R-mediated inhibition of forskolin-induced β-galactosidase activity yields a pA₂ value of 7.8, which is in agreement with the hH₄R affinity of JNJ 7777120 (Table 2.4) (Jablonski et al., 2003; Thurmond et al., 2004). At the mouse and rat H₄R, 4-methylhistamine also acts as a full H₄ agonist, although with reduced pEC₅₀ values of respectively 5.8 ± 0.1 and 5.6 ± 0.1.

Previously the H₄R has been shown to be involved in the regulation of eosinophil and mast cell function (O’Reilly et al., 2002; Buckland et al., 2003; Hofstra et al., 2003; Ling et al., 2004; Takeshita et al., 2004; Thurmond et al., 2004). Indeed, 4
methylhistamine also acted as H₄R agonist at human eosinophils and induced a rapid change in eosinophil cell shape, as measured by the gated autofluorescence forward scatter assay (Ling et al., 2004). The H₄R agonist 4-methylhistamine acted as a full agonist and dose dependently induced a change in forward scatter (Figure 2.6A) with an EC₅₀ value of 0.36 ± 0.09 μM, which was 3 fold less active compared to histamine. The effects of 4-methylhistamine on human eosinophils were not inhibited by H₁R antagonist mepyramine or H₂R antagonist ranitidine, but could be antagonized by the H₄R antagonist JNJ 7777120 (Figure 2.6B). Finally, 4-methylhistamine was tested as H₄R agonist at mouse bone marrow derived mast cells (BMMC) as described before (Hofstra et al., 2003). Like histamine, 4-methylhistamine dose-dependently induced migration of murine BMMCs with an EC₅₀ value of 12 μM (Figure 2.6B), again a somewhat lower potency compared to histamine (Hofstra et al., 2003).

Figure 2.6. Physiological effects of 4-methylhistamine on leukocyte cells in vitro. (A) 4-Methylhistamine increases human eosinophil shape change, (B) the effect of 4-methylhistamine on human eosinophil shape change is inhibited by H₄R antagonist, but not by H₁R or H₂R antagonist, and (C) 4-methylhistamine dose-dependently increase migration of murine bone marrow mast cells (BMMC).
DISCUSSION

With the addition of the H₄R to the histamine receptor family (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001), this potential new drug target has created a lot of excitement in the field. The predominant expression of the histamine H₄R on hematopoietic cells (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001) and the H₄R effects on e.g. eosinophil and mast cell functions (Ganter et al., 2002; O'Reilly et al., 2002; Buckland et al., 2003; Hofstra et al., 2003; Ling et al., 2004; Takeshita et al., 2003; Thurmond et al., 2004) implies that this new histamine receptor subtype may play a role in various allergic and inflammatory conditions. So far, the search for of selective H₄R ligands has resulted in the discovery of potent neutral H₄R antagonists as JNJ 7777120 (Jablonowski et al., 2003; Thurmond et al., 2004) and VUF 6002 (Terzioglu et al., 2004), whereas potent and selective H₄R agonists or inverse agonists have so far not been described. In search for new hH₄R ligands, we therefore screened a library of known histaminergic ligands, using SK-N-MC cells stably expressing the hH₄R. In this cell line the H₄R binds [³H]histamine and [³H]JNJ 7777120 with high affinity (Figure 2.1A and 2.1B) and functionally inhibits forskolin-induced CRE-mediated responses through pertussis toxin-sensitive Gᵢₒ proteins (Figure 2.1D). In these cells the hH₄R also exhibits constitutive activity, which is blocked by pertussis toxin or the non-selective inverse agonist thioperamide (Figure 2.1D).

Considering the high sequence similarity with the H₃R, it is not surprising that the H₄R is targeted by various imidazole containing H₃R ligands (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001)). The standard H₃R inverse agonist thioperamide (Arrang et al., 1983) also acts as an inverse agonist at the H₄R. Moreover, in the present study we confirm that the presumed H₃R agonists immepip, imetit, (R)-α-methylhistamine, and imbutamine also act as potent H₄R agonists. Furthermore, the H₄R is activated by the H₂R/H₃R antagonist burimamide and the H₃R antagonists as clobenpropit and iodoproxyfan, indicating that for H₄R agonism considerable structural diversity (piperidine, isothiourea, thiourea, ether) in the side chain is allowed, including aromatic substitutions, as indicated by the H₃R
agonism displayed by clobenpropit. However, our detailed analysis of various H₃R ligands indicates that H₄R efficacy can be modulated by differential aromatic substitution of the side chain. In the burimamide series we observed that differential substitution of the thiourea group gives rise to H₄R (partial) agonists, a neutral antagonist (VUF 4614, pKᵢ = 7.6) and a full inverse agonist (VUF 4742, pKᵢ = 6.9). Also, in the clobenpropit series, we observe that a slight change of the isothiourea substituent results in a modulation of H₄R efficacy. The clobenpropit analog iodophenpropit (a phenyl substitutent instead of a benzyl group) retains high H₄R affinity (pKᵢ = 7.9), but has lost agonistic activity completely. In this study we identified iodophenpropit as a high affinity, neutral antagonist for the H₄R. In view of the ~15 nM affinity of iodophenpropit for the hH₄R, we evaluated [¹²⁵I]iodophenpropit as a potential new H₄R radioligand. The hH₄R can be labeled to the same extent with both the agonist [³H]histamine and the neutral antagonist [³H]JNJ 7777120. Surprisingly, the Bmax value determined with [¹²⁵I]iodophenpropit was twice as much as that determined with either [³H]histamine or [³H]JNJ 7777120, suggesting that the three radioligands bind to hH₄R subpopulations. The binding of the three radioligands to membranes of SK-N-MC/hH₄ cells was displaced by a variety of H₃/4R ligands and the pKᵢ values obtained from these displacement studies show a high correlation. Despite being showed as a potential hH₄R radioligand, [¹²⁵I]iodophenpropit has to be used with caution, as in our hands a high level of non-specific binding limits its use.

Based on our findings that also iodoproxyfan has a high affinity (pKᵢ = 7.9) the observation of Corbel et al. (1997) of [¹²⁵I]iodoproxyfan binding to a non-H₃R binding site on the myeloid cell line FDCP-2 is of interest. The [¹²⁵I]iodoproxyfan binding to a proposed histamine transporter might actually represent [¹²⁵I]iodoproxyfan binding to the hH₄R (Corbel et al., 1997).

From our screening of many H₁R ligands only the tricyclic clozapine shows reasonable H₄R affinity, as reported before (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). Despite their structural similarity to clozapine, the tested H₁R antagonists do not show any appreciable affinity for the hH₄R. We can therefore
not confirm that mepyramine binds to the hH_{4}R (Nguyen et al., 2001), either studied by displacement of \[^{3}H\]histamine binding to the hH_{4}R, by saturation \[^{3}H\]mepyramine binding assays (data not shown), or by functional H_{4}R assays. Clinically used H_{1}R antagonists, such as cetirizine, ebastine, fexofenadine, and loratidine, demonstrate significant \textit{in vitro} anti-inflammatory activity, which are not related to their H_{1}R activity (Gefland et al., 2004). The data from our study do not support the involvement of the hH_{4}R in the anti-inflammatory effects of these H_{1}R antagonists.

An important finding of this study is the discovery of 4-methylhistamine as a potent and selective hH_{4}R agonist in both recombinant and endogenously expressing H_{4}R systems. Whereas this compound is originally described as a relatively selective H_{2}R agonist (Durant et al., 1975), our present data show that this histamine analog exhibits more than 100-fold selectivity over the recombinant H_{1}R, H_{2}R, and H_{3}Rs. 4-Methylhistamine not only acts as a full agonist at the recombinant hH_{4}R, but also induces migration of mouse bone marrow derived mast cells and a shape change of human eosinophils. Both processes have recently been shown to be induced by histamine via interaction of the H_{4}R (Hofstra et al., 2003; Ling et al., 2004). Similar to the observations with histamine (Hofstra et al., 2003; Ling et al., 2004), the potency of 4-methylhistamine on human eosinophils and the mouse mast cells is somewhat lower than in recombinant system. Although the mouse H_{4}R shows a lower affinity for both histamine and 4-methylhistamine compared to the hH_{4}R, the low potency at BMMC seems not merely an issue of species difference, but might also be related to a low H_{4}R expression level. In fact, the H_{4}R in BMMC is present at a very low density as it can not be detected by radioligand binding studies (Thurmond, unpublished observations). Moreover, the cellular environment might dictate the potency of an agonist, such as composition of G proteins and accessories proteins in the cells (Kenakin, 2004).

In conclusion, from a large screening of many known histamine receptor ligands we have identified a variety of compounds with interesting H_{4}R activities. Based upon our data, many imidazol-containing H_{3}R ligands, including various H_{3}R reference
4-methylhistamine, a potent and selective H₄R agonist

compounds show potent H₄R activities and should be treated with caution. More recently developed H₃R agonists, like immethridine (Kitbunnadaj et al., 2004) or methimmepip (2005) or non-imidazole H₃R antagonists, like JNJ 6379490 (Daugherty, 2004) or A-349821 (Esbenshade et al., 2004), hardly act at the H₄R and will therefore provide good tools to selectively target the H₃R. In the series of tested H₃R ligands, we have identified iodophenpropit as potent neutral H₄R antagonist and the burimamide analog VUF 4742 as the second identified H₄R inverse agonist. From the screening of H₂R ligands we have identified 4-methylhistamine as the first high affinity H₄R agonist (Kᵢ = 50 nM) that has a >100-fold selectivity for the hH₄R over the other histamine receptor subtypes. The identification of 4-methylhistamine as a potent H₄R agonist will be of major importance for future studies to unravel the physiological roles of the H₄R.

MATERIALS AND METHODS

Materials. Burimamide oxalate and burimamide analogs (Vollinga, 1995), clobenpropit dihydrochloride, dimaprit dihydrobromide, histaprodifen dimaleate, homohistamine dihydrobromide, imbutamine dihydrobromide, imetit dihydrobromide, impenta mine dihydrobromide, immepip dihydrobromide, immethridine dihydrobromide, JNJ 7777120, methimmepip dihydrobromide, 2-pyridylethylamine (PEA) dihydrochloride, 2-(2-thiazolyl)ethylamine (TEA), thioperamide fumarate, and VUF 8328 (S-[2-(4-imidazolyl)propyl]isothiourea dihydrobromide) were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam. Ketotifen fumarate and 8R-lisuride were purchased from ICN Biomedicals Inc. (USA), amoxapine, d-chlorpheniramine maleate, clozapine, cimetidine, N-desmethyl clozapine, diphenhydramine hydrochloride, doxepin hydrochloride, forskolin, histamine dihydrochloride, imipramine hydrochloride, loxapine, mepyramine (pyrilamine maleate), (R)-α-methylhistamine dihydrochloride, (S)-α-methylhistamine dihydrochloride, Nβ-methylhistamine dihydrochloride, N-oxide clozapine, octoclothepin, pertussis toxin, polyethyleneimine (PEI), tripeplennamine hydrochloride, and triprolidine hydrochloride were purchased from Sigma RBI (USA). 2-Nitrophenol-β-D-pyranoside (ONPG) and G418 were from Duchefa (The Netherlands), and [³H]Nα-methylhistamine (85 Ci/mmol), [³H]histamine (12.4 Ci/mmol), and [³H]mepyramine (23 Ci/mmol) from Perkin-Elmer Life Science, Inc. (USA). [¹²⁵I]iodoaminopotentidine and [¹²⁵I]iodophenpropit were labeled at the Department Nuclear Medicine and PET Research, Vrije Universiteit Medical Centre, Amsterdam, as described previously (Jansen et al., 1992), while [³H]JNJ 7777120 (84 Ci/mmol) was synthesized at Johnson & Johnson Pharmaceuticals, Inc., USA (Thurmond et al., 2004). Gifts of astemizole (Janssen Pharmaceutica NV, Belgium), cyproheptadine hydrochloride (MSD, The Netherlands), cetirizine hydrochloride (UCB Pharma, Belgium), ebastine (Almirall Prodesfarma, Spain), loratidine (Schering Plough, USA), mianserin hydrochloride and Org 3770 (Organon NV, The
Netherlands), mizolastine (Synthelabo, France), proxyfan dihydrochloride and iodoproxyfan dihydrochloride (Dr. J.A.M. Christiaans (Kovaleinen et al., 1999)), R(+) and S(-)-terfenadine carboxylate (Sepracor, Inc., USA), 2-methylhistamine dihydrochloride, 4-methylhistamine dihydrochloride, and impromidine dihydrochloride (SmithKline, Beecham, UK), are greatly acknowledged.

**Cell culture.** SK-N-MC cell lines, which stably express either the human H<sub>3</sub>R (SK-N-MC/hH<sub>3</sub>) or H<sub>4</sub>R (SK-N-MC/hH<sub>4</sub>) as well as a cAMP responsive element (CRE)-driven β-galactosidase reporter gene SK-N-MC/hH<sub>3</sub> or SK-N-MC/hH<sub>4</sub> cells (Lovenberg et al. 1999; Liu et al., 2001a), were cultured in EMEM medium supplemented with 5% fetal calf serum, 0.1 mg/ml streptomycin, 100 u/ml penicillin, and 600 μg/ml G418 at 37ºC in 5% CO<sub>2</sub> and 95% humidity.

**Radioligand binding assays.** The SK-N-MC/hH<sub>3</sub> cell homogenates were incubated for 40 minutes at 25ºC with approximately 1 nM [³H]N<sup>α</sup>-methylhistamine in 25 mM KPO<sub>4</sub> buffer and 140 mM NaCl (pH 7.4 at 25ºC), with or without competing ligands, whereas the SK-N-MC/hH<sub>4</sub> cell homogenates were incubated 1 hour at 37ºC in 10 nM [³H]histamine and 50 mM Tris-HCl (pH 7.4 at 37ºC), with or without competing ligands. Bound radioligands were collected on 0.3% polyethyleneimine pretreated Whatmann GF/C (and washed three times with 3 ml of ice-cold washing buffer (4ºC) containing 25 mM Tris-HCl and 140 mM NaCl (pH 7.4 at 4ºC) for the hH<sub>3</sub>R and 50 mM Tris-HCl (pH 7.4 at 4ºC) for the hH<sub>4</sub>R. Binding analysis of 10 nM [³H]JNJ 7777120 and 0.1 nM [¹²⁵I]iodophenpropit to the hH<sub>4</sub>R was performed with the same conditions as described for [³H]histamine. In saturation binding analysis, the nonspecific binding of [³H]histamine or [³H]JNJ 7777120 was determined with 1 μM clobenpropit. The binding analysis of [³H]mepyramine and [¹²⁵I]iodoaminopotentidine binding to human H<sub>1</sub>R and human H<sub>2</sub>R, respectively, was performed according to Bakker et al. (2004). The binding data were analyzed with Prism 4.0 (Graphpad Software, Inc.) and data are presented as mean ± SEM. Mouse and rat H<sub>4</sub>R radioligand binding assays was performed according to Liu et al. (2001b).

**Colometric cyclic AMP Assay.** A reporter CRE-β-galactosidase reporter gene assay was employed to determine (inverse) agonistic or antagonistic activity of either the hH<sub>3</sub>R or hH<sub>4</sub>R. Approximately 4 million cells/96-well plate of SK-N-MC/hH<sub>3</sub> and SK-N-MC/hH<sub>4</sub> cells were exposed for six hours to histaminergic ligands in serum-free EMEM medium containing 1 μM forskolin. Thereafter, the medium was discarded, the cells were lysed in 100 μl assay buffer (100 mM sodium phosphate buffer at pH 8.0, 4 mM ONPG, 0.5% Triton X-100, 2 mM MgSO<sub>4</sub>, 0.1 mM MnCl<sub>2</sub>, 40 mM β-mercaptoethanol), incubated overnight at room temperature, and the β-galactosidase activity was determined at of 420 nm with a PowerwaveX340 plate reader (Bio-Tek Instruments, Inc., USA).

**Primary cell experiments.** Cell culture of BALB/c mice-derived bone marrow mast cells (BMMC) and in vitro BMMC chemotaxis assay was performed as previously described (Hofstra et al., 2003). Purification of human polymorphonuclear leukocytes (PMNL) and the human eosinophil shape change assay were performed as previously described (Ling et al., 2004).
CHAPTER 3

Oligomerization of recombinant and endogenously expressed human histamine H₄ receptors

Van Rijn RM¹ and Chazot PL¹, Shenton FC, Sansuk K, Bakker RA, Leurs R,
Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry,
Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
Centre for Integrative Neuroscience (CINS), School of Biological & Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, England

Adapted from Molecular Pharmacology 2006, 69(4):1194-206
"Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved."
ABSTRACT

In this study we report the homo- and hetero-oligomerization of the human histamine H₄R (hH₄R) by both biochemical (Western blot and immobilized metal affinity chromatography) and biophysical (BRET and tr-FRET) techniques. The H₄R receptor is the most recent discovered member of the histamine family of G protein-coupled receptors. Using specific polyclonal antibodies raised against the C-terminal tail of the H₄R we demonstrate the presence of H₄R oligomers in HEK 293 and COS-7 cells heterologously overexpressing H₄Rs, as well as putative native H₄R oligomers in human PHA blasts endogenously expressing H₄Rs. Moreover, we show that H₄R homo-oligomers are formed constitutively, are formed at low receptor densities (300 fmol/mg protein) and are present at the cell surface, as detected by tr-FRET. The formation of these oligomers is independent of N-glycosylation and not modulated by H₄R ligands, covering the full spectrum of agonists, neutral antagonists and inverse agonists. Whereas we show H₄R homo-oligomer formation at physiological expression levels, the detection of H₁R-H₄R hetero-oligomers was achieved only at higher H₁R expression levels and are most likely not physiologically relevant.

INTRODUCTION

The human histamine H₄R (hH₄R), a prototypical member of the superfamily of G-protein coupled receptors (GPCRs), has been identified recently through the use of bioinformatics by several groups simultaneously.¹⁴⁸⁻¹⁵² The H₄R couples to members of the Gᵢ/ₒ family of heterotrimeric G-proteins to mediate the inhibition of adenylyl cyclase. In addition, the receptor may activate phospholipase C and induce calcium mobilization.¹⁹⁴ The H₄R expression is almost exclusively restricted to hematopoietic cells and is suggested to mediate functions of the immune system. As such the H₄R is a target for the development of anti-inflammatory drugs.¹⁶⁴,¹⁷²,¹⁹⁴
The use of various biochemical and biophysical approaches has recently revealed that members of the GPCR family may exist as homo- as well as hetero-oligomers at the cell surface. When considering the hetero-trimeric G protein, which is about twice the size of the GPCR, it seems reasonable that GPCRs need to oligomerize to interact with the G protein as suggested for the leukotriene B4 receptor. Currently, hetero-oligomerization has been shown to be pivotal for the GABAβ1R1, which needs to associate with GABAβ2 receptors in order to be transported to the cell membrane, and for the T1R taste receptors, which require hetero-oligomerization to form receptors that can recognize sweets or amino-acids. In other cases, hetero-oligomerization may change the ligand binding characteristics potentially giving rise to a new dimension in GPCR drug discovery.

We have previously reported the detection of homo-oligomers of the human histamine H1 receptor (H1R) by applying biochemical and tr-FRET experiments, as well as by the formation of H1R radioligand binding sites upon the co-expression of two ligand-binding deficient mutant H1Rs. The H1R is a well known target for the treatment of seasonal allergies, but has also been shown to mediate inflammatory responses in keratinocytes. The H1R is ubiquitously expressed, and is co-expressed together with the H4R in leukocytes, including monocytes and T lymphocytes, suggesting that on these cells histamine may modulate inflammatory actions through the action on both H1Rs and H4Rs. We therefore investigated the potential homo-oligomerization of the H4R as well as the hetero-oligomerization of the H1R with the H4R using heterologous expression systems.

Herein, we report on the generation of specific antibodies raised against the H4R, the detection of homo-oligomers of the H4R, and the potential formation of H1R-H4R hetero-oligomers by using biochemical as well as BRET and tr-FRET approaches. Using these methodologies, we show the human H4R to constitutively form homo-oligomers at the cell surface, and that the oligomerization is independent of ligand stimulation of the receptors. Furthermore, N-glycosylation of the H4R receptor is not a pre-requisite for oligomer formation. Whereas we can detect H4R homo-oligomers at physiologically relevant H4R expression levels and
in endogenously H₄R expressing PHA blast cells, the detection of H₁R-H₄R hetero-oligomers requires higher receptor expression levels.

RESULTS

Pharmacological characterization of the hH₄R and hH₁R expressed in COS-7 cells

We have previously used COS-7 cells successfully for the heterologous expression of the hH₁R as well as for the identification of H₁R oligomers. To investigate the potential oligomerization of the human H₄R (hH₄R) we therefore expressed the hH₄R heterologously in COS-7 cells. Transfection of these cells with cDNA coding for the hH₄R resulted in the expression of a high affinity [³H]histamine binding site (Table 3.1). Subsequent displacement studies using [³H]histamine as a radioligand revealed these binding sites to display a characteristic H₄R pharmacological profile (Table 3.1). The H₁R and H₄R constructs used in the tr-FRET, BRET and immobilization assays were also characterized by radioligand binding (saturation and displacement) assays. COS-7 cells were transiently transfected with cDNA encoding the HA-H₄R, FLAG-H₄R, H₄R-Rluc, H₄R-eYFP, c-myc-H₄R-his, HA-H₁R, H₁R-Rluc, or the H₁R-eYFP. [³H]histamine bound to the H₄Rs according to a one site saturable model with Hill slopes of approximately 1 and dissociation constants (Kₑq) similar to the wild-type hH₄R, although the Bₘₐₓ value is affected by fusion of the Renilla luciferase enzyme (Table 3.1). Bound [³H]histamine could be displaced from all the N- and/or C-terminally tagged H₄Rs by the agonist histamine and the inverse agonist thioperamide with affinity values (pKᵢ) comparable to the wild-type (Table 3.1). Similarly the H₁R radioligand [³H]mepyramine bound the various hH₁R constructs according to a one site saturable binding model with Kₑq values similar to the wild-type hH₁R (data not shown). The agonist histamine and H₁R inverse agonist mepyramine were able to displace the radioligand with affinities equal to the wild-type H₁R (data not shown). The aforementioned H₄R constructs were functionally characterized using a CRE-luciferase-luciferase reporter gene assay. In these assays histamine behaved as a full H₄R agonist and thioperamide as a full
inverse H₄R agonist for each H₄R-construct with pEC₅₀ values comparable to those obtained for the wild-type H₄R (Table 3.1).

Table 3.1. Characterization of epitope-tagged and H₄R fusion constructs. The Kᵦ values for histamine in nM, expression (B_max) levels in pmol/mg protein, and the pKᵦ values of histamine and thioperamide for the H₄R constructs used in the experiments were determined by [³H]histamine saturation and displacement binding assays. The pEC₅₀ values of histamine and thioperamide as determined using a CRE-luciferase reporter gene assay. The values are expressed as mean ± SEM of at least three separate experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kᵦ</th>
<th>B_max</th>
<th>Histamine</th>
<th>Thioperamide</th>
<th>pEC₅₀</th>
<th>Histamine</th>
<th>Thioperamide</th>
<th>pIC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type H₄R</td>
<td>19.9 ± 1.4</td>
<td>1.0 ± 1.4</td>
<td>7.6 ± 0.1</td>
<td>7.3 ± 0.1</td>
<td>8.8 ± 0.2</td>
<td>6.4 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA-H₄R</td>
<td>23.2 ± 0.6</td>
<td>2.0 ± 0.5</td>
<td>7.4 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>8.9 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLAG-H₄R</td>
<td>26.3 ± 0.5</td>
<td>1.3 ± 0.3</td>
<td>7.6 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>9.0 ± 0.1</td>
<td>6.5 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₄R-Rluc</td>
<td>30.8 ± 2.3</td>
<td>0.1 ± 0.2</td>
<td>7.6 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>9.0 ± 0.1</td>
<td>6.0 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₄R-eYFP</td>
<td>57.4 ± 3.6</td>
<td>1.1 ± 0.3</td>
<td>7.2 ± 0.1</td>
<td>7.3 ± 0.2</td>
<td>8.6 ± 0.3</td>
<td>5.7 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-myc-H₄R-his₁₀</td>
<td>33.3 ± 2.0</td>
<td>2.6 ± 0.5</td>
<td>7.3 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>9.0 ± 0.1</td>
<td>6.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Generation of hH₄R specific antibodies

To enable our biochemical approaches and to study H₄R function in native tissue we raised a rabbit polyclonal anti-hH₄ (374-390) receptor antibody, which represents the first published selective immunological probe for the hH₄R. The antibody was generated against the last 17 amino acids of the C-terminal tail of the H₄R (Figure 3.1A). The selectivity of the anti-hH₄R antibody was confirmed by blockade with the C-terminal peptide of the H₄R (Figure 3.1B, lane 3) and a lack of cross reactivity with the human H₃R, the most related GPCR (Figure 3.1B, lane 1). In transfected HEK 293 cells, the antibody detects two major reactive species at Mₙ 34-36 kDa and 65-72 kDa (Figure 3.1B, lane 2). The lower bands most likely represent monomeric H₄Rs. Occasionally an addition band (Mr approx. 45 kDa) was detected which is likely to be a proteolytic fragment. We suspect the Mₙ 34 kDa to be the unglycosylated product of the species at Mₙ 36 kDa. The higher molecular weight species, could either represent a heavily glycosylated form of the H₄R or dimeric H₄Rs.
Figure 3.1. Characterization of specific polyclonal H₄R antibodies. A, snake plot of the hH₄R; the region of the C-terminal tail (374-390) against which the antibody was raised is marked in grey. HEK 293 cells expressing hH₃(445)R or hH₄R (B), and human PHA blasts (C) were probed by immunoblotting using the anti-H₄ (374-390) antibody (0.5 µg /ml) either alone or pre-incubated for 16h at 4°C with 500 µg /ml (374-390) peptide. The major immunoreactive species labeled in the HEK 293 hH₄R and the human PHA blasts were greatly suppressed by preincubation with the antigen peptide (B, lane 3 and C, lane 2, respectively), demonstrating the sequence selectivity of the antibody. Furthermore, no significant labeling of the hH₃(445)R (B, lane 1) or in untransfected HEK293 cells (not shown) was detected.
Evidence that native H₄R are robust dimers

The H₄R clearly plays a role as an immune modulator, with mRNA expression shown in e.g. human mast cells, neutrophils, eosinophils and T lymphocytes. A single major diffuse immunoreactive species (approx. Mr 77 kDa) coincident with the putative recombinant dimeric hH₄R species expressed in COS-7 cells was detected in human PHA blasts (Figure 3.1C, lane 1). This species was abolished by preincubation with the (374-390) peptide, again demonstrating the peptide selectivity of the antibody. Interestingly, little or no protein monomers were detected in the native preparation, consistent with our previous data with the H₃R. Notably, these experiments were performed under reducing conditions, indicating the robust nature of the dimeric species in native tissue. An identical labeling pattern was detected with the anti-hH₄ 374-390 antibody probing human spleen lysates (not shown). The putative dimeric recombinant hH₄R species expressed in HEK 293 cells was consistently smaller (approx. Mr 72 kDa which may reflect differential glycosylation in the two cell lines (Figure 3.3). Coincident protein species were detected by the anti-hH₄ 374-390 and the anti-epitope-tagged antibodies in the respective cell lines, further confirming that the hH₄ receptor is being labeled by the anti-hH₄ 374-390 antibody (not shown). No signal was detected in either COS-7 or HEK 293 cell lines, further supporting the selectivity of the anti-hH₄ 374-390 antibody. These data identifies for the first time the H₄R protein in human T lymphocytes.

Immunoprecipitation of recombinantly expressed HA-H₄Rs from HEK 293 cells

To further characterize the selectivity of the H₄R antibody an immunoprecipitation assay was performed. HA-H₄Rs transiently expressed in HEK 293 cells (Figure 3.2, Lane 3-5) were immunoprecipitated using anti-HA antibodies (Figure 3.2, lane 4), or a non-immune Ig (Figure 3.2, lane 3). As negative control non transfected HEK 293 cells (Figure 3.2, lane 1), and non transfected HEK293 cells immunoprecipitated with anti-HA antibodies (Figure 3.2, lane 2) were used. As positive control solubilized HEK 293 cells expressing HA-H₄Rs (Figure 3.2, lane 5) was used. All samples were subjected to immunoblotting using the anti-H₄R
antibodies. Immunoreactive species were only detected for the HEK 293 cells expressing the HA-H4R, which had been anti-HA immunoprecipitated (Figure 3.2, lane 4). The immunoreactive species represent the putative monomeric and dimeric H4R and are identical to the reactive species in the positive control (Figure 3.2, lane 5).

Figure 3.2. The anti-H4R antibodies recognize anti-HA immunoprecipitated HA-H4Rs. HEK 293 cells alone (lane 1-2) or transfected with cDNA encoding the HA-H4R (lane 3-5) were subjected to immunoprecipitation with an anti-HA antibody (lane 2, 4) or a non-immune Ig (lane 3). The precipitates (lane 2-4) or solubilized cells (lane 1, 5) were immunoblotted using the anti-H4R antibody.

Cross linking of H4Rs
To further investigate the homo-oligomeric structure of the H4R, a crosslinking study was performed using N-terminally c-myc-tagged H4R expressed in COS-7 cells.
Upon application of increasing concentrations of the cell impermeable crosslinker BS$_3$, a progressive reduction in the monomeric doublet species ($M_c$, 34 kDa and 36 kDa) was observed (Figure 3.3A). Concomitant the appearance of, initially, a diffuse species of $M_c$, 77 kDa (putative glycosylated and unglycosylated dimers) and, then, higher molecular weight species ($M_c$, > 175 kDa) at 0.25mM and 2mM BS$_3$, respectively was noticed (Figure 3.3A). These data are highly consistent with hH$_4$Rs expressed in HEK 293 cells (data not shown).

**Biochemical evidence that the hH$_4$Rs is an N-glycosylated homo-dimer**

In the N-terminus of the hH$_4$R Asn$^5$ and Asn$^9$ are potential sites for N-glycosylation (Figure 3.3B). To study whether the higher molecular weight species are the N-glycosylated forms of the hH$_4$R, we expressed H$_4$Rs in the presence of the N-glycosylation inhibitor tunicamycin. In the absence of tunicamycin, two major putative monomeric species, $M_c$, 34 kDa and $M_c$, 36 kDa, and a diffuse $M_c$, 65-72 kDa species were detected as in Figures 3.1 (see also Figure 3.3C, lane 1). In the presence of 2 µg/ml tunicamycin, a complete loss of the $M_c$, 36 kDa species and concomitant increase in intensity of the $M_c$, 34 kDa species and an additional species at $M_c$, 32 kDa was observed (Figure 3.3C, lane 2). Furthermore, the diffuse $M_c$, 65-72 kDa species, detected in the absence of tunicamycin, was reduced to a single $M_c$, 65 kDa species. Notably, an increase in tunicamycin concentration had no further effect upon either the $M_c$, 34 kDa or $M_c$, 65 kDa species (Figure 3.3C, lanes 3-5). The $M_c$, 32 kDa species observed upon tunicamycin treatment is likely to be a breakdown product of the glycosylated $M_c$, 36 kDa species in untreated cells. These data strongly suggest, that the recombinant hH$_4$R is N-glycosylated and forms dimers. This last process is independent of post-translational N-glycosylation.

Notably, upon enzymic N-deglycosylation of PHA blasts, the Mr 77 kDa species was greatly reduced in intensity, and a new Mr 34 kDa species was detected, consistent with the monomeric hH$_4$R (Figure 3.3D, lane 2).
Figure 3.3. Evidence for hH4R dimers and higher oligomers and glycosylation of the hH4R dimers. A, COS-7 cells transfected with cDNA encoding the hH4R were subjected to cross-linking using increasing concentrations of BS3 (0.12-2 mM). The resultant pellets were subjected to immunoblotting and probed with the anti-hH4 (374-390) antibody. Lane 1, COS-7 cells expressing hH4Rs as control; Lanes 2-5, COS-7 cells expressing hH4Rs treated with 0.12, 0.5, 1 and 2 mM BS3, respectively. The * species is likely to be a proteolytic fragment of the hH4R (observed in both host cells). B, Snakeplot of the N-terminal tail and beginning of TM1 of the H4R; arrows are directed to possible N-glycosylation sites. C, HEK 293 cells transfected with the hH4R were grown in the absence and presence of 2, 4, 6 and 8 µg/ml tunicamycin for 48h. The cells were harvested, homogenates prepared and subjected to immunoblotting. Immunoblots were probed with the anti-hH4 (374-390) receptor antibody. Lane 1, hH4Rs in absence of tunicamycin; Lanes 2-5, hH4Rs in presence of 2, 4, 6 and 8 µg/ml tunicamycin, respectively. D, PHA blasts were subjected to N-deglycosylation with PNGase F enzyme. Control PHA blasts were incubated in parallel with deglycosylation buffer alone. Samples were then subjected to immunoblotting, and immunoblots probed with the anti-hH4 (374-390) receptor antibody. Lane 1, control; Lane 2, in the presence of PNGase F. Enzymatic deglycosylation resulted in the reduction in intensity of the Mr 77 kDa species and appearance of the Mr 34 kDa putative monomer.
**HA-H₄Rs associate with c-myc-H₄R-his₁₀**

To further investigate if the H₄Rs can associate with each other to form homo-oligomers, membranes of COS-7 cells co-expressing N-terminally c-myc and C-terminally his₁₀ tagged hH₄Rs (c-myc-H₄R-his₁₀) and N-terminally HA-tagged hH₄Rs (HA-H₄R) were solubilized and loaded on a Ni²⁺-resin column. The HA-H₄Rs, when co-expressed with the c-myc-H₄R-his₁₀, were retained on the Ni²⁺-column and could be eluted with 250 mM imidazole as detected with anti-HA antibodies (Figure 3.4, lane 3). When cells individually expressing c-myc-H₄R-his₁₀ and HA-H₄Rs were mixed prior to solubilization, and subsequently loaded on the column, no HA-H₄Rs were found to interact with the Ni²⁺-resin (data not shown).

**Figure 3.4.** Biochemical detection of homo-dimeric H₄Rs. Cells co-expressing H₄Rs with an N-terminal c-myc- and C-terminal His₁₀-tag (c-myc-H₄R-his₁₀) and an N-terminally HA-tagged H₄Rs (HA-H₄R) receptors were solubilized and loaded onto a Ni²⁺-NTA column. Samples were taken of the solubilized receptors before loading onto the column (lane 1), of the unbound fraction (lane 2) and of the bound fraction, that was eluted using 250 mM imidazole (lane 3). Samples were resolved by SDS-PAGE and then immunoblotted using anti-HA antibodies.

**BRET shows constitutive ligand-independent homo-oligomerization of hH₄Rs**

The use of biophysical techniques has been of great value to the study of GPCR oligomerization. We have used BRET to study in further detail the homo-oligomerization of the H₄R. BRET was performed on COS-7 cells expressing either the H₄R-Δluc or co-expressing the H₄R-Δluc with the H₄R-eYFP. After addition of
coelenterazine a robust BRET signal could be observed in the cells co-expressing the two H_4Rs (Figure 3.5B). As a negative control, cells individually expressing either of the H_4R constructs were mixed before adding coelenterazine (Figure 3.5B). Previous studies have reported the ability of H_1Rs to oligomerize^{199,205}. Therefore cells in which the H_1R-Rluc and the H_1R-eYFP were co-expressed were taken as positive control. In these cells, a BRET signal was detected, that was approximately 2 fold lower than that observed for the H_4Rs (Figure 3.5B).

To investigate the effect of ligands on H_4R oligomerization, cells co-expressing the H_4R-Rluc with the H_4R-eYFP were incubated with 10 μM of the agonist histamine, the neutral antagonist iodophenpropit^{115} or the inverse agonist thioperamide for 15 minutes before the actual BRET measurement. No significant change was observed in BRET signal between stimulated and non stimulated cells (Figure 3.5C).

Agonist-induced increase in oligomerization of somatostatin receptors occurs at physiological expression levels (160 fmol/mg protein) but not after overexpression^{206}. We therefore also tested the effect of histamine stimulation on H_4R oligomerization at different expression levels. While maintaining H_4R-Rluc expression level constant (around 0.2 pmol/mg protein) we reduced the amount of expressed H_4-eYFP. The concomitant reduction of the donor/acceptor ratio resulted in an expected drop in BRET signal. At total H_4R expression levels, of 1.0, 0.6 or 0.3 pmol/mg a significant BRET signal was observed. However, histamine did also not effect the H_4R oligomerization at lower H_4R expression levels (Figure 3.5D).
Figure 3.5. Evaluation of homo-oligomerization of the H4R and homo-oligomerization of the H1R by BRET using the co-expression of Renilla luciferase (Rluc) and eYFP C-terminal receptor-fusion proteins. A, Schematic representation of BRET. After addition, coelenterazine is converted by the (Rluc) enzyme fused to the C-terminus of a receptor into light of a wavelength of 470 nm, which when in close proximity (<100Å) can excite the eYFP protein fused to the C-terminus of another receptor leading to the emission of light at a wavelength of 530 nm B, BRET ratios for the hH4R homo-oligomers compared with the hH1R homo-oligomers. Cells expressing the indicated receptor-fusion proteins were exposed to 5 μM coelenterazine after which energy transfer was measured. Cells individually expressing either H4R-Rluc or H4R-Rluc were mixed prior to exposure to coelenterazine with cells individually expressing H4R-eYFP or H4R-eYFP, respectively (open bars). C, Effects of a 15 minutes stimulation of 10 μM histamine (Hist), iodophenpropit (IPP) or thioperamide (TP) on the BRET ratios for the hH4R homo-oligomers. D, Effects of a 15 minute stimulation of 10 μM histamine (Hist) on H1Rs homo-oligomers. Cells were expressed with a constant amount of H4R-Rluc (~ 0.2 pmol/mg protein) and a decreasing amount of H4R-eYFP. Total H4R expression was 1.0, 0.6 and 0.3 (pmol/mg protein). Ratios are expressed as mean ± S.E.M. from at least three experiments each performed in triplicate.
**tr-FRET shows presence of hH₄R oligomers at cell surface.**

To study whether the H₄Rs oligomers are actually present at the cell surface we performed tr-FRET assays on COS-7 cells co-expressing N-terminally FLAG tagged H₄Rs (FLAG-H₄R) and HA tagged H₄Rs (HA-H₄Rs). These cells were incubated with Europium (Eu³⁺) labeled anti-FLAG antibodies or a combination of the Eu³⁺ labeled anti-FLAG and allophycocyanin (APC) labeled anti-HA antibodies. As control, cells individually expressing the FLAG-H₄Rs and the HA-H₄Rs were mixed and exposed to the two antibodies. Only from the cells co-expressing the FLAG-H₄Rs and HA-H₄Rs a pronounced signal was observed (Figure 3.6B). This FRET signal can only be explained due to the resonance energy transfer from Eu³⁺ anti-FLAG antibodies bound to FLAG-H₄Rs to APC anti-HA antibodies bound to HA-H₄Rs. Since this resonance energy transfer can only take place within 100Å, the data indicates the formation of H₄R oligomers at the cell surface of living cells. Stimulation of the COS-7 cells with 10 μM histamine or 10 μM thioperamide preceding tr-FRET measurement did not result in a significant change in signal (Figure 3.6B). The used antibodies did not have an influence on the ligand binding to the H₄Rs as no significant difference was found in [³H]histamine binding in the absence or presence of the antibodies (data not shown).

**Lack of hetero-oligomerization between H₄R and H₁Rs.**

We have subsequently used tr-FRET to investigate whether hetero-oligomerization occurs between H₄Rs and H₁Rs. tr-FRET was performed on COS-7 cells co-expressing the FLAG-H₄Rs and N-terminally HA-tagged histamine H₁Rs (HA-H₁Rs). As control, cells individually expressing the FLAG-H₄Rs and the HA-H₁Rs were mixed and exposed to the two antibodies. No significantly increased tr-FRET signal could be observed compared to the signal obtained from cells individually expressing the two receptors that were mixed prior to incubation with the antibodies (Figure 3.6C). The ratio and total amount of antibodies was maintained equal between experiments with H₁R-H₄Rs and H₄R-H₁Rs to ensure proper comparison.
Figure 3.6. Evaluation of homo-oligomerization of the H₄R and hetero-oligomerization of the H₄R with the H₁R by tr-FRET using co-expression of differentially epitope-tagged receptors. A, Schematic representation of tr-FRET. Excitation at 337 nm of anti-FLAG Eu⁺³ antibody bound to the FLAG-epitope tagged receptor leads to emission of light at a wavelength of 620 nm, which when in close proximity (<100Å) can excite the anti-FLAG APC antibody bound to another FLAG-epitope tagged receptor leading to the emission of light at a wavelength of 665 nm. B, tr-FRET using cells co-expressing FLAG-tagged and HA-tagged H₄Rs. Cells were incubated for 2 hours with the Eu⁺³-labelleled anti-FLAG antibodies (open bar, Eu⁺³), or with both Eu⁺³-labelleled anti-FLAG and APC-labelleled anti-HA antibodies (filled bars, APC) in the presence or absence of 10 μM histamine (Hist) or 10 μM thioperamide (Thiop). C, tr-FRET using cells co-expressing FLAG-tagged H₄Rs (FLAG-H₄R) and either HA-tagged H₄Rs (HA-H₄R) or HA-tagged H₁Rs (HA-H₁R). Cells were incubated for 2 hours with the Eu⁺³-labelleled anti-FLAG antibodies (open bar, Eu⁺³), or with both Eu⁺³-labelleled anti-FLAG and APC-labelleled anti-HA antibodies (filled bars, APC). Data are normalized for the tr-FRET signal obtained from a mixture of cells that was obtained by mixing of cells that have been incubated with Eu⁺³-labelleled anti-FLAG antibodies with cells that have been incubated with APC-labelleled anti-FLAG antibodies. Data shown are from a representative experiment.

Comparable results were obtained using Eu⁺³ anti-HA antibodies and APC anti-FLAG antibodies (data not shown). Stimulation of co-transfected cells with 10 μM histamine did not lead to a change in FRET signal (data not shown).
Homo-oligomerization of H4Rs vs hetero-oligomerization of H4Rs and H1Rs.

To further investigate hetero-oligomerization between H4Rs and H1Rs, BRET saturation curves were produced for both the H4R homo-oligomer and the H4R-H1R hetero-oligomer. Experiments were performed in which COS-7 cells were transfected with a fixed amount of H4R-Rluc and increasing amounts of either H4R-eYFP or H1R-eYFP cDNA. Expression levels were determined by radioligand binding. Expression of the H4R-Rluc was maintained around 0.2 pmol/mg protein while. Expression levels of H4R-Rluc were correlated with luminescence and expression levels for the eYFP fused H1R and H4R were correlated with fluorescence.

Figure 3.7. Evaluation of receptor-expression dependency of the detection of H4R homo-oligomers and H4R-H1R hetero-oligomers using BRET. BRET saturation curves for the hH4R homo-oligomers (H4R-Rluc + H4R-eYFP, solid line) compared with H1R-H4R hetero-oligomers (H4R-Rluc + H1R-eYFP, dotted line) at increasing expression levels of the eYFP tagged receptor. COS-7 cells were transfected with a fixed amount DNA encoding for the H4R-Rluc and increasing amounts of DNA encoding for the H4R-eYFP or the H1R-eYFP. Plotted on the X-axis is the fluorescence obtained from the eYFP, which has been correlated to the expression of H4R-eYFP (●) and H1R-eYFP (○). Expression level of the H4R-Rluc was maintained around 200 (fmol/mg protein) as determined from the luminescence, which has been correlated to the expression of the H4R-Rluc.
A linear correlation was obtained for all three constructs. Expression for the H4R-eYFP ranged from 0.3-2.5 pmol/mg protein, whereas expression levels of the H1R-eYFP ranged from 0.5-16 pmol/mg protein. For the H4R homo-oligomers a steep increase in BRET signal is observed, showing detectable BRET when total H4R expression is 0.3 pmol/mg protein. For the H1R-H4R hetero-oligomers a more gradual increase in BRET signal is observed upon increased expression of the H1R-eYFP. A BRET signal is observed for the first time at expression levels of 1 pmol/mg protein of the H1R-eYFP. The H4R-H4R homo-oligomer showed a two fold lower BRET50 (0.77 vs 1.6) and a 2.5 fold higher Bmax (0.1 vs 0.04) than the H1R-H4R hetero-oligomer as determined from the BRET saturation curve. (Figure 3.7).

DISCUSSION

GPCR oligomerization has become a generally accepted phenomenon and has been reported to occur in all GPCR classes. Data obtained from atomic force microscopy, electron microscopy and western blot analysis has provided compelling evidence that the light sensitive rhodopsin is predominately present as a dimer in the retinal disc membrane. For histamine receptors oligomerization has been convincingly shown for the hH1Rs, the hH2Rs and hH3Rs. In view of the emerging role of GPCR oligomerization in GPCR function and our interest in the H4R as new target for inflammatory conditions, we have investigated oligomerization of the human H4R by various means. Combining biophysical measurements like tr-FRET and BRET with biochemical approaches, like Western blot analysis and histidine-tag based affinity chromatography, we provide compelling evidence for homo and hetero-oligomer formation of hH4Rs.

To enable our biochemical approaches and to study H4R function in native tissues, we report in this study on the first polyclonal H4R antibody that can successfully be used for Western blot analysis. This new molecular tool is directed against the C-terminal tail of the H4R and detected monomeric and potential dimeric H4R species after Western blot analysis of membranes from HEK 293 and COS-7 transfected cells. The selectivity of the new H4R antibody was confirmed by blockade with the C-terminal peptide, used to raise the antibody and the lack of cross reactivity.
towards the highly related human hH₄R. Furthermore, through an immunoprecipitation study the H₄R antibody was shown to detect the same HA-H₄Rsb as detected by a commercially available anti-HA antibody

Western blot analysis of the H₄R expressed in tunicamycin treated cells, indicate that the H₄R normally is N-glycosylated, most likely at Asn⁵ and/or Asn⁹ of the extracellular N-terminus of the H₄R. However, inhibition of N-glycosylation did not affect the presence of putative dimeric H₄R species on the Western blot. To show that H₄R proteins are in close proximity of each other, a requirement for oligomerization, a cross linking experiment, using BS₃ was performed. With increasing concentrations of the crosslinker BS₃, the bands representing the monomeric H₄R disappeared. At the same time, bands representing oligomeric H₄Rsb became more apparent. These data indicate that the H₄Rsb are in close enough proximity for cross linking by BS₃ and suggests that the 65-72 kDa species might represent dimerized H₄R proteins. Finally, the polyclonal H₄R antibody allowed us to study the presence of H₄R proteins in human PHA blasts. High level H₄R mRNA expression has been shown in various white blood cells, including T-lymphocytes, but H₄R protein expression has so far not been shown. Western blot analysis of membranes of PHA blasts with our polyclonal anti-H₄R antibody, indeed revealed the presence of H₄R protein in PHA blasts. Interestingly, the endogenously expressed H₄R was only detected as high molecular weight species. Enzymatic deglycosylation of the native H₄R protein resulted in a partial reduction of the high molecular weight species (77 kDa) to monomeric H₄Rsb (34 kDa). These data in itself do not directly exclude a heavily glycosylated (ca.33 kDa) H₄R protein. However the hH₄Rsb in human HEK293 cells is only moderately glycosylated (ca. 2 kDa) and the high molecular weight species coincide with the putative dimeric H₄Rsb when recombinantly expressed in COS-7 (77 kDa) and HEK293 cells (72 kDa). These data can be explained by assuming that in human PHA blasts the hH₄R functions predominantly as a dimer. We hypothesize that N-glycosylation is not a prerequisite for dimerization, but it helps to stabilize the H₄R dimers. A similar stabilizing effect of glycosylation on receptor dimers has recently been shown for the human bradykinin B₂ receptors. The putative H₄R dimerization in native tissue clearly warrants further investigation.
As an alternative biochemical method, we employed an immobilized metal (Ni\textsuperscript{2+}) affinity chromatography approach with a histidine-tagged H\textsubscript{4}R protein. To this end, we co-expressed c-myc-H\textsubscript{4}R-his\textsubscript{10} and HA-H\textsubscript{4}Rs receptors to study oligomer formation via affinity column chromatography. In contrast to c-myc-H\textsubscript{4}R-his\textsubscript{10} receptors, HA-H\textsubscript{4}Rs are not robustly retained onto a Ni\textsuperscript{2+}-NTA resin when expressed alone. Yet, c-myc-H\textsubscript{4}R-his\textsubscript{10} receptors immobilized onto Ni\textsuperscript{2+}-NTA resin were shown also to retain co-expressed HA-H\textsubscript{4}Rs on the Ni\textsuperscript{2+}-NTA column, as determined by HA-immunoreactivity detected after elution of histidine-tagged proteins with high imidazole concentrations. These findings indicate that c-myc-H\textsubscript{4}R-his\textsubscript{10} receptors physically interact with the co-expressed HA-H\textsubscript{4}Rs to form oligomers that can be retained on the Ni\textsuperscript{2+}-NTA resin through the C-terminal his\textsubscript{10} tag.

We continued our investigation of the oligomerization of the H\textsubscript{4}Rs in living cells using BRET and tr-FRET assays. Using the BRET assay, a clear signal could be detected when co-expressing H\textsubscript{4}R-Rluc with H\textsubscript{4}R-eYFP in non-stimulated cells, suggesting constitutive homo-oligomerization of H\textsubscript{4}Rs. Since the oligomers detected in the immobilization- and BRET-assay do not necessarily have to be present at the cell surface, we also studied H\textsubscript{4}R oligomerization on the cell membrane of living cells by tr-FRET. The tr-FRET approach uses antibodies, which do not permeate the cell membrane, and detects cell surface H\textsubscript{4}R oligomers present at the cell surface. Similar to the BRET assay, we detected a robust signal, indicating constitutive presence of H\textsubscript{4}R homo-oligomers at the cell surface.

A number of studies have investigated the effect of agonists on receptor oligomerization. However, at present the effects of ligand-stimulation on GPCR oligomerization are not consistent. It has been found that agonists can promote or reduce GPCR oligomerization, or are without effect on GPCR oligomerization.\textsuperscript{39,207,213} In the case of the H\textsubscript{4}R, we did not detect any significant difference in BRET signal if cells were treated with either the H\textsubscript{4}R agonist histamine, the neutral H\textsubscript{4}R antagonist iodophenpropit\textsuperscript{115} or the inverse H\textsubscript{4}R agonist thioperamide\textsuperscript{115,150}, suggesting that H\textsubscript{4}R ligands do not modulate H\textsubscript{4}R homo-oligomerization. Similarly, no agonist or inverse agonist induced modulation of H\textsubscript{4}R oligomerization was detected in the tr-FRET assay. Previously, Patel et al. reported that agonist-
induced oligomerization of somatostatin receptors was only detected at physiological expression levels, but not after over-expression. We therefore performed BRET experiments at various H₄R expression levels. First of all, it is interesting to notice that already at an H₄R expression level of approximately 300 fmol/mg protein significant BRET signals can be observed. These data indicate that at physiological expression levels the H₄R can indeed homo-oligomerize and corroborate our findings of H₄R dimers, detected on PHA blasts with our anti-H₄R antibody. Secondly, from the BRET experiments we might also conclude that also at low expression levels of the H₄Rs homo-oligomerization is not affected by agonist stimulation. Nevertheless, one should be aware that results concerning ligand effects on dimerization obtained with these biophysical assays can be difficult to interpret, since agonist-induced changes in H₄R conformation could potentially influence the energy transfer between the energy-acceptor and -donor.

The H₄R has been linked to play a role in inflammation based on its expression pattern and recent findings, showing that the H₄R induces chemotaxis of eosinophils and mast cells, and stimulates the release of IL-16 from CD8⁺ T cells, and the release of leukotriene B₄ in zymosan-challenged mice. Interestingly, the H₁R is co-localized with the H₄R in several white blood cells and also plays a prominent role in inflammatory conditions. Since both the H₁Rs and the H₄R (this study) are able to form homo-oligomers, we were prompted to study whether the H₄Rs can form hetero-oligomers with the H₁Rs. In fact, previous work with hetero-oligomeric opioid receptors has revealed that GPCR hetero-oligomerization brings an additional layer of complexity to the class of GPCR proteins, but also offers opportunities to develop hetero-oligomeric selective ligands (Waldhoer et al., 2005). Yet, using tr-FRET assays, we were unable to detect H₁R-H₄R hetero-oligomers, suggesting that such GPCR hetero-oligomers are not present at the cell surface. In contrast to the tr-FRET experiments, we were able to detect an expression-level dependent formation of H₁R-H₄R hetero-oligomers using BRET. Distinct from the detection of H₄Rs homo-oligomers, H₁R-H₄R hetero-oligomers were only detected at high expression levels, and we failed to detect hetero-oligomers at physiologically relevant conditions.
Results from BRET saturation studies demonstrate a higher propensity for the formation of H₄R-H₄R homo-oligomers over H₁R-H₄R hetero-oligomers. We presume that the signal observed with BRET at high expression levels possibly originates from intracellular H₁R-H₄R hetero-oligomers. Whereas some receptors, such as the 5-HT₁A receptor appear to readily form heteromeric receptors, our present H₄R data corroborate the idea that GPCR hetero-oligomerization is highly selective, as reported for instance for the adrenergic receptors and the thyrotropin-releasing hormone receptors. Although hetero-oligomerization has been shown to occur even between receptors from different classes, the relatively low homology (23%) between H₁R and H₄Rs is apparently too low to readily form hetero-oligomers.

In conclusion, we have developed specific antibodies against the C-terminus of the H₄R, which allowed the detection of endogously expressed H₄R proteins. This anti-hH₄R antibody will be an important new molecular tool to study the localization and function of the H₄R. Moreover, we determined by various methods that the H₄R constitutively forms cell-surface homo-oligomers. Homo-dimeric H₄Rs are not only found using heterologous expression systems, but are also present in PHA blasts and spleen lysates, endogenously expressing H₄Rs. The formation of H₄R oligomers is not dependent on N-glycosylation, nor affected by ligand stimulation but possibly destabilized by deglycosylation. While H₁R-H₄R hetero-oligomers could be detected using BRET upon receptor overexpression, these hetero-oligomers are most likely not present at the cell surface. Moreover, H₁R-H₄R hetero-oligomers were not found at physiologically relevant expression levels. Future studies will have to reveal if the H₁R can form hetero-oligomers with other GPCR family members or preferentially exists as homo-oligomer.

**MATERIALS AND METHODS**

**Materials.** Reagents for tr-FRET were from Cis bio international (Bagnols-sur-Cèze Cedex, France). Coelenterazine was purchased from Chemicon international (Temecula, CA, USA). Sheep anti-mouse IgG horseradish peroxidase (HRP) was from Amersham bioscience (Little Chalfont, Buckinghamshire, England). Bovin serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine(2-[4-imidazolyl]ethylamine hydrochloride), mepyramine (pyrilamine maleate), monoclonal mouse anti-FLAG (DYKDDDDK), polyethyleneimine was purchased from Sigma (USA). Calf serum (Integro BV,
Dieren, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Life technologies (Merelbeke, Belgium). Cell culture plastics were from Greiner Bio-one (Wemmel, Belgium). Tris was from AppliChem (Darmstadt, Germany). [3H]Histamine (12.40 and 18.10 Ci/mmol) and [3H]mepyramine (23.00 Ci/mmol) were purchased from Perkin-Elmer Life Science, Inc. (USA). Oligonucleotides were purchased from Isogen Biocience (Maarsen, The Netherlands). PfuTurbo® DNA polymerase was purchased from Stratagene (La Jolla, CA). Restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). Thioperamide, iodophenpropit, clobenpropit and JNJ 7777120 (1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine), were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands. Gifts of mouse anti-hemagglutinin (anti-HA) antibody (Dr. J. van Minnen), pcDNA3.1-eYFP vector (Dr. T. Schmidt), pRL-CMV vector (Dr. G. Milligan), pCR3.1-HA-H1R and pcDEF3-c-myc-H-R (Dr. S Hill), expression vector pcDEF3 (Dr. J Langer,21), and mianserin (Organon NV) are greatly acknowledged. Wild type human H4R in pcDNA3.1 was purchased from Guthrie cDNA resource center (Sayre, PA). The vector was subcloned into the pcDEF3 using BamHI/XbaI sites.

**Construction of epitope-tagged proteins for tr-FRET.** An N-terminally FLAG (DYKDDDDK) epitope tagged H4R was created by PCR. The coding sequence of the H4R gene was amplified using the following sense oligonucleotide primer 5’-GGGAAGCTTGGCCACCATGGACTACAAGAGCGATGACAAGGATCCAGATACTAATAGCAC-3’ and antisense primer 5’-GGAAAGGCACGGGGAGGGC-3’. The amplified gene was first cloned into the pCRII-Topo vector by TOPO TA cloning® (Invitrogen BV, Breda) and subsequently subcloned into the pcDEF3 expression vector using EcoRV/XbaI sites.

An N-terminally hemagglutinin HA (YPYDVPDYA) epitope tagged H4R was created by PCR in two steps. The H4R gene was amplified by PCR with a 5’ SacII site and without start codon using the following sense primer 5’-ACCGCGGCCCCAGATACTAATAGCACACGAC-3’ and antisense primer 5’-GGAAAGGCACGGGGAGGGGAGGAC-3’. The amplified gene was first cloned into the pCRII-Topo vector. The gene was subsequently subcloned using SacII/XbaI sites into the pcDNA3.1-HA-rH3AR vector204. The HA-H4 gene was finally subcloned using BamHI/XbaI sites to pcDEF3.

The HA-H4R gene was subcloned from the pCR3.1-HA-H1R into the pcDEF3 using Bsp1407I/SpeI restriction sites.

**Construction of fusion proteins for BRET.** For the BRET assay H4Rs were c-terminally fused to either a Renilla luciferase (H4R-Rluc) or a yellow fluorescent protein (H4R-eYFP) in two steps. The coding sequence of the H4R gene was amplified without its stop codon using the following sense primer 5’-TGGATCCACATGGACATGACTAATAGC-3’ and antisense primer 5’-CGCGCGGCCGACTAGTAGAAGATACTAGCCGAC-3’, harboring unique BamHI and NotI restriction sites, respectively. The gene was cloned directly into the pCRII-Topo vector and subsequently subcloned to a pcDEF3 vector using BamHI/NotI sites (pcDEF3-H4R (Del stop)).

The coding sequence for the Rluc gene was amplified from the pRL-CMV vector lacking a start codon and harboring a NotI restriction site using the following sense primer 5’-
The coding sequence for the eYFP gene was amplified from the pcDNA3.1-eYFP vector lacking a start codon and harboring a NotI restriction site using the following sense primer 5'-CGCGGCCGCCTGAGCAAGGGCGAGAG-3' and antisense primer 5'-TCTAGAATTGTTCTAGCTGCGCAG-3'. The gene was directly cloned to the pCRII-Topo vector and subsequently subcloned in frame using NotI/XbaI sites into the pcDEF3-H4R (Del stop) vector.

A hH1R-eYFP fusion was generated by PCR using the following sense primer 5'-AAGAGAATTCCTGGATCCATGGTGAGCAAGGGCG-3' and antisense primer 5'-TTCTCTAGATTCTGTACAGCTGAGCTCCATG-3', harboring unique EcoRI and XbaI restriction sites, using pcDNA3.1eYFP as template. The PCR fragment was digested using EcoRI and XbaI and the purified fragment was subsequently ligated together with the fragment that was obtained by digestion of the pcDEF3-hH1R plasmid using EcoRI/XbaI sites. A hH1R-Rluc fusion was generated by PCR using the following sense primer 5'-AAGAGAATTCCTGGATCCATGGTGAGCAAGGGCG-3' and antisense primer 5'-CGCTCTAGATTCTGTACAGCTGAGCTCCATG-3', harboring unique EcoRI and XbaI restriction sites, using. The PCR fragment was digested using EcoRI and XbaI and the purified fragment was subsequently ligated together with the fragment that was obtained by digestion of the pcDEF3-hH1R plasmid using EcoRI/XbaI sites. Each construct was fully sequenced before its expression and analysis.

**Construction of His10-tagged proteins for immobilization.** An N-terminally c-myc (EQKLISEEDL) and C-terminally His10-epitope tagged H4R was created as follows. First a c-myc epitope tagged H4R was created by PCR in two steps. The c-myc tag was amplified by PCR using a pcDEF3-c-myc-H4R vector as template with a 3' NheI site using the following sense 5'-GGGTGGAGACTGGATAGGCC-3' and antisense primer 5'-GTGCTAGCAGGTCTTCCTGCGC-3'. The fragment was directly cloned to the pCRII-Topo vector (pCRII-topo-myc). The H4R gene was amplified without start codon and containing a 5' Nhel restriction site using the following sense 5'-CCGCTAGCCATGGTGAGCAAGGGCG-3' and antisense primer 5'-TCTTTAAGAAGATACTGAGCGAC-3'. The gene was directly cloned to the pcDNA3.1/V5-His-Topo vector. The H4R gene was subsequently subcloned in frame using Nhel/NcoI into the pCRII-topo-c-myc vector (pCRII-topo-c-myc-H4R). The c-myc-H4R gene was subsequently subcloned into the pcDEF3 expression vector using the BamHI/XbaI sites. Second the gene of the wild type H4R was amplified by PCR without start and stop codon with a 5'BamHI site and a 3' SpeI site using the following sense 5'-CCGGATCCCTGAGCATACCTAGCCTTTAACTAATAGCAC-3' and antisense primer 5'-CCGGCGCCGACTTAATAGCAATATAGCAC-3' and directly cloned into the pcDEF3-Topo vector. The H4R gene was than subcloned in frame from the pCRII-topo-vector using BamHI/SpeI sites in the pSFV2genB vector. An N-terminally tagged FLAG and C-terminally tagged H4R-His10 gene was subcloned from the pSFV2genB-FLAG-H4R-His10 behind the p10 promoter of the pFastbac_DUAL vector using Ncol/Nhel restriction sites.
The H₄R-His₁₀ gene was amplified by PCR from the pFastbac_DUAL-FLAG-H₄R-His₁₀ vector without start codon and a 3' XbaI site using the following sense 5'-CATCTAGATTAATTACCCACTGGGCC-3' and antisense primer 5'-GAGGATCCGCCAGATACTAATAGCACAATC-3' and directly cloned into the pcDNA3.1/V5-His-Topo vector by TOPO TA cloning and subsequently subcloned into the pcDEF3-c-myc-H₄R vector using BsmI/XbaI restriction sites. Each construct was fully sequenced before its expression and analysis.

**Cell culture and transfection of COS-7 cells.** COS-7 African green monkey kidney cells were maintained at 37°C humidified in 5% CO₂/95% air atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 50 IU/ml of penicillin and 50 μg/ml streptomycin and grown in 100 mm dishes. Cells were transiently transfected using the DEAE-dextran method as previously described. The total amount of DNA transfected was maintained constant by addition of pcDEF3.

**Cell culture and transfection of HEK 293 cells.** HEK 293 cells were maintained at 37°C humidified in 5% CO₂/95% air atmosphere in DMEM/F12 (Cambrex, UK) supplemented with 10% (v/v) fetal calf serum, 50 IU/ml of penicillin and 50 μg/ml streptomycin and grown in 100 mm dishes. HEK 293 cells were transfected with pcDEF3-H₄R receptor essentially using the LipofectaminePlus method described in Shenton *et al.*, 2005. Briefly for each cDNA, two microtubes were prepared: tube 1 contained 2µg cDNA, 6µl of lipofectaminePLUS reagent (Invitrogen) and 150µl of Optimem-I media (Gibco); tube 2 contained 5µl lipofectamine reagent (Invitrogen) and 150µl of Optimem-I media. The mixtures were incubated at room temperature for 15 minutes after which the contents of tube 2 were added to tube 1 followed by a further 15 minute incubation. In the meantime the HEK 293 cells at 50-80% confluence in 2ml petri dishes were washed three times with Optimem-I media. At the end of the second incubation period the contents of tube 1 were made up to 1.5ml with Optimem-I media and added to the washed HEK 293 cells. The cells were incubated at 37°C for 6 hours. The transfection mixture was then removed and replaced with growth media. The cells were harvested 48 hours post-transfection, and cell homogenates prepared for immunoblotting.

**[³H]histamine binding studies.** Cells used for radioligand binding studies were harvested 48h post transfection and homogenized in ice cold H₄R binding buffer (50 mM tris, pH 7.4). For saturation isotherms cell membrane homogenates were incubated at 37°C for 60 minutes with 0-125 nM [³H]histamine in a total assay volume of 200 μl. Non-specific binding was determined by incubation in the presence of 10 μM JNJ 7777120. For competition binding assays the cell homogenates were incubated at 37°C for 60 minutes with 0.1-10.000 nM ligand in the presence of ~15 nM [³H]histamine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice cold H₄R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filterplates that had been treated with 0.3% polyethyleneimine. Filters were washed four times with H₄R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.
Oligomerization of H₄R

[³H]mepyramine binding studies. Cells used for radioligand binding studies were harvested 48 hours post transfection and homogenized in ice cold H₁R binding buffer (50 mM Na₂/K-phosphate buffer, pH 7.4). For saturation isotherms cell membrane homogenates were incubated at room temperature for 30 minutes with 0-25 nM [³H]mepyramine in a total assay volume of 200 μl. Non-specific binding was determined by incubation in the presence of 1 μM mianserin. For competition binding assays the cell homogenates were incubated at room temperature for 30 minutes with 0.1-10.000 nM in the presence of ~1.5 nM [³H]mepyramine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice cold H₁R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filterplates that had been treated with 0.3% polyethyleneimine. Filters were washed four times with H₁R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

Anti-H₄R antibody generation. The unique peptide corresponding to the amino acids CIKKQLPSQHRSVSS of the human H₄R subtype was conjugated to thyroglobulin by the Cys-coupling method. The resultant conjugate was used to generate polyclonal antibodies in rabbits. Antibody production and affinity purification was performed as previously described.

Production of human PHA blasts. Human peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (PHA blasts) were generated essentially as previously described. Briefly, heparinized human whole blood was obtained from healthy volunteers (with Local ethical approval), and PBMCs were separated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway) and centrifuged at 400 X g for 25 min. The PBMCs were isolated from the interfacial layer, washed twice in RPMI without L-glutamine (Gibco™) and resuspended in RPMI complemented with 10% (v/v) Fetal Calf Serum, 1% (v/v) Penicillin, and Streptomycin, and 1% (v/v) L-Glutamine. Cell density was adjusted accordingly to 1 x 10⁶ cells/ml with RPMI. 100μl PHA (‘Lectin’, Sigma, UK) was added to the cells to make PHA Blasts. These were grown in culture for 24h, harvested and a cell homogenate prepared in the presence of protease inhibitors (Protease Inhibitor Cocktail III, Calbiochem, UK).

Immunoblotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 6 % or 7.5 % polyacrylamide slab gels under reducing conditions. Samples of HEK 293 cells, COS-7 cells and PHA blasts (20-50 μg protein) were prepared using a chloroform/methanol method of protein precipitation, and immunoblotting was performed as previously described. Immunoblots were probed with anti-H₄ 374-390 antibody at a concentration of 0.5 μg /ml. Blots containing FLAG or c-myc tagged receptors were probed with primary antibodies, mouse anti-FLAG (1.5 μg/ml) or mouse anti-c-myc (1 μg/ml), respectively. Horseradish peroxidase conjugated goat anti-mouse antibodies (1:2000-5000) were used as secondary antibodies.

Immunoprecipitation HEK 293 cells were transfected with HA-H₄ receptor, and solubilized with 1% Triton X100/0.15M NaCl for 30 min at 4°C. Immunoprecipitation was performed essentially as previously described. Solubilized HEK 293 cell extracts were incubated with 5 μg of rat anti-HA antibody (Roche
Diagnostics, Germany) or rat non-immune Ig (ADI, USA) at 4°C for 1 hour. 50 µl of pre-chilled washed Protein G agarose (Sigma, UK) slurry was added and incubated for 1 hour at 4°C on a rocking platform. Precipitation pellets were collected by centrifugation at 10,000xg for 30 seconds at 4°C, washed with 3 x PBS, resuspended in sample buffer, vortex-mixed and heated to 90-100°C for 3 minutes. The sample was then re-centrifuged, and the supernatant subjected to immunoblotting. Control experiments were performed using untransfected HEK 293 cells.

Cross-linking experiments. The cross-linking method used was essentially as described by Shenton et al. 204,210. Briefly, aliquots of COS-7 cells expressing c-myc-H4Rs were pelleted, the suspension buffer was removed and replaced with 150µl cross-linking buffer (150mM NaCl, 100mM Na-HEPES, 5mM EDTA pH7.5, 5mM DTT) to give a final protein concentration of approx. 0.5mg/ml. The cross-linker (bis(sulphosuccinimidyl) suberate sodium salt) was dissolved in 20mM HCl to give a 100mM stock solution. The tubes were incubated at room temperature with continual mixing for 12 minutes with 0.25, 0.5, 1.0 and 2 mM crosslinker, centrifuged at 10,000rpm for 5mins, the cross-linking mixture removed and the resultant pellet prepared for immunoblotting.

Tunicamycin experiments. HEK 293 cells expressing H4Rs were incubated with 2, 4, 6 and 8 µg/ml tunicamycin (stock dissolved in DMSO at 2 mg/ml) immediately after transfection and harvested 48 h post-transfection, homogenized and subjected to immunoblotting. 225. Cells grown in the absence of tunicamycin were incubated with the respective volume of DMSO.

Deglycosylation of native H4 receptor. Human PHA blast cell suspensions were resuspended in deglycosylation buffer (50µM sodium phosphate pH6.0, containing 0.1% SDS, 0.1% β-mercaptoethanol and 20mM EDTA) and incubated with either water (control) or PNGase F enzyme (Sigma, UK) at a final enzyme concentration of 400iu/ml (test) for 16 hours at 37°C. The samples were then subjected to immunoblotting and probed with anti-H4 374-390 antibody at a concentration of 2µg/ml. The NMDAR1 transfected into HEK 293 cells was used as a positive control essentially as described in 226.

Receptor immobilization. Membranes of COS-7 cells transiently expressing c-myc-H4R-His and FLAG-H4R or HA-H1R-His and FLAG-H4R receptors were homogenized, solubilized and subsequently immobilized on Ni-NTA columns (Invitrogen) as previously described 204. Immobilized receptors were eluted using 250 mM imidazole. Samples were prepared for immunoblotting subjected to chloroform/methanol extraction loaded on a 7.5 % SDS page gel and subsequently blotted on nitrocellulose paper (Amersham).

BRET assay. Forty-eight hours post-transfection cells were detached with trypsin and washed twice with PBS. Approximately 50,000 cells per well were distributed in white bottom 96-wells microplates (Corning BV, Schiphol-Rijk). Coelenterazine was added to a final concentration of 5 µM and readings were collected immediately following this addition using a Victor2 allowing signal detection at 460 nm and 530 nm.
**tr-FRET assay.** tr-FRET assays were performed using Eu³⁺-labeled and allophycocyanin anti-FLAG and anti-HA antibodies as described by 204. Briefly, tr-FRET was assessed in 1 x 10⁶ whole COS-7 cells transiently expressing the appropriate HA and FLAG tagged receptors. Cells were incubated in PBS containing 50% FCS (v/v), 0.8 nM of Eu³⁺-labelled antibody, and 8 nM of allophycocyanin-labelled antibody for two h at room temperature on a rotating wheel, after which the membranes were washed twice with PBS. The final pellet was resuspended in 50 μl PBS and transferred to a 384-microtiter plate. Energy transfer was measured by exciting the Eu³⁺ at 320 nm and monitoring the XL-665 allophycocyanin emission for 500 μs at 665 nm using a Novostar (BMG Labtechnologies) configured for time-resolved fluorescence after a 100 μs delay.

**Analytical methods.** Binding data were evaluated by a non-linear least squares curve fitting program using Graphpad Prism® (Graphpad Software Inc, San Diego, CA). Protein concentrations were determined according to Bradford 227, using BSA as standard. All data are represented as mean ± S.E.M. from at least three independent experiments in triplicate. Statistical significance was determined by a Students unpaired t-test (p<0.05 was considered statistically significant).
CHAPTER 4

Cloning and characterization of dominant negative splice variants of the human histamine H4 receptor

Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
School of Biological & Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, England
Leiden University Medical Center Skin Research Lab, Einthovenweg 20, 2300 RC Leiden, The Netherlands
UMR 8147, Centre National de la Recherche Scientifique, Faculte de Medecine Rene Descartes, Paris V, Hopital Necker
ABSTRACT

The histamine H₄ receptor (H₄R) is the latest identified member of the histamine receptor subfamily of G protein-coupled receptors with potential functional implications in inflammatory diseases and cancer. The H₄R is primarily expressed in eosinophils and mast cells and shares highest homology with the H₃R. The occurrence of at least twenty different hH₃R isoforms led us to investigate the possible existence of H₄R splice variants. Herein we report on the cloning of the first two alternatively spliced H₄R isoforms from CD34+ cells from cord blood. These H₄R splice variants are expressed in mast cells and eosinophils and are localized predominantly intracellular when expressed recombinantly in mammalian cells. We failed to detect any ligand binding, H₄R-ligand induced signaling or constitutive activity for these H₄R splice variants. However, when co-expressed with the full-length H₄R (H₄R(390)), the H₄R splice variants have a dominant negative effect on the surface expression and function of the H₄R(390). We detect H₄R(390)– H₄R splice variant hetero-oligomers by employing both biochemical (immunoprecipitation, cell-surface labeling) and biophysical (time-resolved FRET) techniques. Messenger RNAs encoding the H₄R splice variants are detected in various cell types and are expressed at similar levels as the full-length H₄R(390) mRNA in e.g. premonocytes. We conclude that the herein described H₄R splice variants have a dominant negative effect on H₄R(390) functionality, being able to retain the H₄R(390) intra-cellularly and to inactivate a population of H₄R(390) presumably via hetero-oligomerization.

INTRODUCTION

The human histamine H₄ receptor (hH₄R) is the fourth and most recently discovered member of the G-protein coupled receptor (GPCR) subfamily of histamine receptors 148-153. The H₄R is predominantly expressed in hematopoietic cells and is suggested to play a role in inflammation 164,168 and allergy 184. Interestingly, recently connections of the H₄R with rheumatoid arthritis 188, colon cancer 189,190 and breast cancer 191 have also been proposed. Consequently, the
H₄R is currently seen as a potential new drug target and is getting much attention from pharmaceutical industry.

The hH₄R gene has been cloned on the basis of its relatively high homology with the histamine H₃ receptor (H₃R). The H₄R gene shows the same genomic organization as the H₃R gene, with the positions of two introns at identical sites as found for the H₃R gene, potentially resulting in the existence of differentially spliced isoforms, as have been detected for both human and rat H₃Rs. A large variety of genes encoding GPCRs have been found to be alternatively spliced and alternative splicing thereby introduces additional diversity to GPCR pharmacology. Natural occurring splice variants may have practically identical pharmacological properties as the full-length receptor, but can also have altered functionality or lack any functional responses. Moreover, the expression of full-length receptors and receptor isoforms can be differentially regulated as was recently demonstrated for e.g. the expression of the neurokinin-1 receptor and a C-terminally truncated neurokinin-1 receptor isoform in undifferentiated and differentiated THP-1 cells.

For the H₄R a large number of alternatively spliced isoforms have been described. Many human H₃R isoforms with deletions in the third intracellular loop and potentially different signal transduction have been reported. However, also a variety of non-7TM isoforms are found in brain mRNA. Recently, we have shown that several natural occurring 6TM rH₃R splice variants interfered with trafficking of the full-length rH₃R to the cell membrane, hence reducing its signaling properties. Such dominant negative behavior of alternatively spliced isoforms has also been described for e.g. the α₁A-adrenergic receptor, the dopamine D₃nde receptor, an alternatively spliced rat vasopressin V₂ receptor, and a naturally occurring splice variant of the calcitonin receptor lacking exon.

In view of the great interest in the H₄R as a pharmacological target and the large diversity of GPCR isoforms for the related H₃R, the identification of H₄R isoforms is of utmost importance for the future understanding of H₄R pharmacology and target validation strategies. In the present study we report on the identification and pharmacological characterization of two new splice variants of the hH₄R. Although translated and present at the cell surface, albeit to a lesser extent than the H₄R(390).
these receptor isoforms are non-functional when individually expressed in recombinant mammalian cells. However, when co-expressed with the hH4R(390) the two H4R isoforms are able to decrease [3H]histamine binding to the hH4R(390) and histamine potency. Surface labeling experiments showed a reduction in H4R(390) surface expression when co-expressed with either of the H4R isoforms. The apparent mechanism underlying the dominant negative effect might be attributed to hetero-oligomerization between the hH4R(390) and the H4R splice variants as determined by biophysical and biochemical techniques.

RESULTS

Cloning of human H4R splice variants.
Using mRNA isolated from mast cells, eosinophils, basophils and dendritic cells derived from CD34+ cord blood cells, we investigated the occurrence of hH4R isoforms. Our RT-PCR approach using specific primers (see Materials and Methods) identified two new H4R splice variants. The first splice variant encodes a protein consisting of 302 amino acids (H4R(302)), compared to 390 amino acids of the H4R(390) and was cloned from mRNA from eosinophils. Alternative splicing of mRNA encoding H4R(302) results in the deletion of exon 2. A previously unidentified exon/intron junction within exon 3 of the hH4R gene, however, allows for the use of an alternative splicing acceptor maintaining the gene in its original frame. Consequently, the H4R(302) isoform has a deletion of 88 amino acids between TMII and TMIV (Figure 4.1). The second splice variant was cloned independently from mRNA from both mast cells and eosinophils. Alternative splicing of mRNA encoding this H4R isoform also results in the removal of exon 2. However, in this case the H4R(390) splice acceptor site of exon 3 is recognized, causing a frame-shift and the introduction of an alternative stop codon. This results in a severely truncated H4R(67) receptor containing only the first 67 amino acids of the hH4R (Figure 4.1).
Figure 4.1. Sequence alignment of human H4R splice variants and genomic arrangement of the H4R gene. A Amino acid alignment of the hH4R with the two identified H4R splice variants. Indicated underneath the amino acids are seven transmembrane domains as proposed for the hH4R(390) (TM1 through TM7). Two arrows indicate where intron 1 and intron 2 is splice out of the H4R(390) mRNA. The H4R(302) splice variant passes over exon 2 and recognizes an alternative acceptor site (arrow between TMIV and TMV) within exon 3. The H4R(67) splice variant also skips exon 2 but recognizes the typical acceptor site after intron 2, resulting in a frameshift and the introduction of an alternative, premature stop codon. B Overview of the exon/intron structure of the H4R gene on chromosome 18q11.2 (Genbank accession number NM_021624 (GI:14251204)). The exon/intron junctions for the H4R(390), H4R(302) and H4R(67) are portrayed. Exon sequences are depicted in upper case, and intron sequences in lower case.

Endogenous occurrence of H4R splice variants mRNA in human cells.
Quantitative PCR (Q-PCR) was subsequently used to investigate the presence of mRNA encoding the genes of the hH4R(390) and the hH4R splice variants in several
Cloning of dominant negative H4R splice variants

cell types. To this end, mRNA of undifferentiated HL-60 cells (pre-monocytes) and HL-60 cells differentiated to produce eosinophils or granulocytes were collected and subjected to Q-PCR analysis. As control, a gene, encoding for ribosomal protein S11 was also amplified to correct obtained mRNA expression levels. RPS11 mRNA expression is not affected by the various experimental conditions used, and Q-PCR efficiency of RPS11 is similar to that of the H4R(390) and the H4R splice variants (data not shown). Of all tested cells, eosinophils expressed the highest amounts of mRNA of the H4R isoforms (Table 4.1). In pre-monocytes the H4R(67) mRNA levels were almost 2-fold higher compared to the H4R(390) mRNA levels, whereas the H4R(320) expression was very low in this cell type. In the other tested cells, the mRNA levels of the H4R(390) were considerably higher compared to the levels of the 2 splice variants (Table 4.1).

Table 4.1. Expression of H4R(390), H4R(302) and H4R(67) messenger RNA in a variety of differentiated HL-60 cells. HL-60 cells were cultured under various conditions (see Materials and Methods) to differentiate the cells to pre-monocytes (pH 7.4 or pH 8.0), granulocytes or eosinophils. The amount of mRNA encoding H4R(390), H4R(302) and H4R(67) were determined by Q-PCR. Messenger RNA expression values were normalized against the validated control gene encoding ribosomal protein S11 (RPS11).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pre-monocytes (pH 8)</th>
<th>Granulocytes</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4R(390)</td>
<td>11.5 ± 10</td>
<td>4.7 ± 1.5</td>
<td>46.8 ± 23</td>
</tr>
<tr>
<td>H4R(302)</td>
<td>&lt;&lt;1</td>
<td>&lt;&lt;1</td>
<td>&lt;&lt;1</td>
</tr>
<tr>
<td>H4R(67)</td>
<td>&lt;1</td>
<td>9.1 ± 5.9</td>
<td>&lt;&lt;1</td>
</tr>
</tbody>
</table>

Fold expression (relative to rps11, *10^-6)

Pharmacological characterization of newly identified H4R splice variants

We have previously employed COS-7 cells successfully for the heterologous expression of the H4R as well as for the identification of H4R oligomers. To study their pharmacology we therefore also expressed the two H4R splice variants heterologously in COS-7 cells. The H4R agonist radioligand [3H]histamine and the H4R antagonist radioligand [3H]JNJ 7777120 bound to the H4R(390) according to a one site saturable model with dissociation constants (Kd) of 23.9 ± 3.6 nM (n=3) and 11.1 ± 3.6 nM (n=3), respectively. The obtained Bmax-values for both radioligands are approximately 1 pmol/mg protein. However, transfection of COS-7
cells with cDNAs coding for the H₄R splice variants did not result in the detection of significant specific radioligand binding using either [³H]histamine (Figure 4.2A) or [³H]JNJ 7777120 (Figure 4.2B).

**Figure 4.2.** The H₄R splice variants are unable to bind the H₄R agonist radioligand [³H]histamine nor the non-imidazole H₄R neutral antagonist radioligand [³H]JNJ 7777120. Saturation isotherms were obtained for [³H]histamine (A) or [³H]JNJ 7777120 (B) by performing radioligand binding on membranes from transiently transfected COS-7 cells expressing either the H₄R(390) (•), H₄R(302) (○) or H₄R(67) (△). A representative experiment is shown.

To determine whether the H₄R splice variants were constitutively active and/or able to signal via Gᵢₒ-proteins, the cDNA encoding the H₄R(390), H₄R(302) or H₄R(67) were heterologously transfected in HEK 293T cells together with a CRE-luciferase reporter gene. Histamine acts as a full agonist (pEC₅₀ = 8.6 ± 0.1, n=5) on the H₄R(390). Moreover thioperamide acts as a full inverse agonist (pEC₅₀ = 6.7 ± 0.1, n=4), revealing the known constitutive activity of the H₄R(390) (Figure 4.3A). No effect of either histamine or thioperamide could be observed for both the H₄R(302) and the H₄R(67) isoforms over a range of 0.1-10.000 nM (Figure 4.3C and 4.3E). Whereas pretreatment with 0.1 µg/ml pertussin toxin (PTX) abolishes the constitutive signaling of the H₄R(390) (Figure 4.3B) in the CRE-reporter gene assay, no effect of PTX was observed for the H₄R(302) and the H₄R(67) isoforms compared to cells not treated with PTX (Figure 4.3D and 4.3F).
Cloning of dominant negative H4R splice variants

Figure 4.3. Functional response of hH4R and H4R splice variants to agonist and inverse agonist stimulation in transiently transfected HEK 293T cells. Dose-response curves for the H4R agonist histamine (●) and H4R inverse agonist thioperamide (○) on the H4R(390) (A), the H4R(302) splice variant (C) and the truncated H4R splice variant H4R(67) (E) in a forskolin (1 μM) induced CRE-luciferase reporter gene assay. Inhibition of Gi-signalling and constitutive activity by pertussis toxin (PTX) pre-treatment of HEK 293T cells expressing the H4R(390) (B), the H4R(302) splice variant (D) and the truncated H4R splice variant H4R(67) (F) were grown for 16 hours in the absence (open bars) or presence (closed bars) of (0.1 μg/ml) PTX. A representative experiment is shown.
**H₄R splice variants are translated and present predominantly intracellularly.** The absence of radioligand binding and Gₛ-coupled signaling of the H₄R splice variants could simply be due to the fact that receptor proteins are not expressed. In order to determine whether the H₄R splice variants are translated into protein and to determine the localization of these splice variants an ELISA was performed with COS-7 cells expressing HA epitope-tagged the H₄R₃₉₀ (HA-H₄R₃₉₀), FLAG epitope-tagged H₄R₃₀₂ (FLAG-H₄R₃₀₂) or FLAG-H₄R₆₇ (Figure 4.4A). The localization (cell surface or intracellular) of the receptors was determined by permeabilization of the cells using NP-40. Whereas the HA-H₄R₃₉₀ is readily observed on the cell membrane, both H₄R splice variants are located predominantly intracellularly, although a small fraction of each was clearly detected on the cell membrane (Figure 4.4A).

**Figure 4.4.** The H₄R splice variants are expressed predominantly intracellular when recombinantly expressed in COS-7 cells. A, ELISA assays were performed on transiently transfected COS-7 cells individually expressing HA-H₄Rs, FLAG-H₄R₃₀₂ or FLAG-H₄R₆₇. Receptor expression and localization were determined using anti-HA and anti-FLAG antibodies on intact (open bars) and permeabilized cells (closed bars). Data has been normalized against COS-7 cells transfected with empty vector. B, HEK 293 cells were transfected with either FLAG-H₄R₃₀₂ or FLAG-H₄R₆₇ receptor isoforms. Intact cells were biotinylated for 15 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin, washed and homogenised. Biotinylated surface fraction was isolated by streptavidin chromatography, and analyzed by immunoblotting using anti-FLAG antibodies. Lanes 1 and 3: Intracellular fraction; Lanes 2 and 4: Surface fractions, where lanes 1 and 2 are FLAG- H₄R₃₀₂ samples, and lanes 3 and 4 and FLAG- H₄R₆₇ samples. Representative experiments are shown.
Surface labeling experiments provided additional evidence for the intracellular localization of the H₄R splice variants (Figure 4.4B). HEK 293 cells expressing the FLAG-H₄R(302) or the FLAG-H₄R(67) were biotinylated to distinguish intracellularly localized H₄R isoforms from receptors present at the cell surface. Similar to the ELISA results, the majority of the H₄R isoforms can be detected intracellularly (Figure 4.4B). In both assays the presence of H₄R splice variants at the cell surface is more evident for the FLAG-H₄R(67) than for the FLAG-H₄R(302) (Figure 4.4B). No anti-FLAG reactivity was observed in MOCK HEK 293 cells (data not shown).

**H₄R splice variants are able to down-regulate H₄R(390) binding.**

Natural occurring splice variants of e.g. the rH₃R have a dominant negative effect on receptor cell-surface expression of the full-length rH₃R. To investigate the effect of the H₄R splice variants on the H₄R pharmacology, [³H]histamine radioligand binding was performed on membranes of COS-7 cells co-expressing the H₄R(390) together with either the H₄R(302) or the H₄R(67) splice variant. [³H]histamine bound to the COS-7 cells co-expressing the H₄R(390) with the H₄R splice variants according to a one site, saturable model with dissociation constants (Kᵰ) similar to the value obtained for the H₄R(390) expressed individually in COS-7 cells (Figure 4.5A, Table 4.2).

**Table 4.2.** Characterization of the hH₄R(390) expressed individually or co-expressed with either the H₄R(302) or the H₄R(67) splice variant in transiently transfected COS-7 cells. The Kᵰ values, the H₄R expression (B_max) and the pKᵢ values of 4-methylhistamine (4-Me-HA), clozapine, JNJ 7777120 and thioperamide were determined by [³H]histamine saturation and displacement binding assays. COS-7 cells were transfected in a 1:1 ratio with cDNA encoding the H₄R(390) and the H₄R splice variants. The values are expressed as mean ± SEM of at least three independent experiments.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kᵰ (nM)</th>
<th>B_max (%)</th>
<th>4-Me-HA</th>
<th>Clozapine</th>
<th>JNJ 7777120</th>
<th>Thioperamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄R(390)</td>
<td>23.9 ± 2.8</td>
<td>100</td>
<td>7.1 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td>H₄R(390) + H₄R(302)</td>
<td>24.4 ± 2.0</td>
<td>45 ± 3</td>
<td>7.1 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>H₄R(390) + H₄R(67)</td>
<td>20.2 ± 1.9</td>
<td>70 ± 5</td>
<td>7.1 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.3 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 4.5. Dominant negative effect of H4R splice variants on [3H]histamine binding to H4R(390). A Saturation of [3H]histamine to H4R receptors expressed individually (●) or co-expressed with the H4R splice variants H4R(302) (□) or H4R(67) (○) in COS-7 cells. B Dose dependent decrease of [3H]histamine binding to H4R(390) by increasing the amount of H4R(302) cDNA transfected transiently in COS-7 cells C Co-expression of H4R(390) with H1R in a 2:1 ratio has no negative influence on H4R(390) binding in COS-7 cells D Displacement of [3H]histamine from H4R expressed individually (●) or co-expressed with the H4R splice variants H4R(302) (□) or H4R(67) (○) in COS-7 cells by the H4R antagonist JNJ 7777120 (dotted line) or H4R agonist clozapine (solid line). E Co-expression of H4R(390) with H4R(302) or H4R(67) in HEK 293T cells decreases histamine potency in a CRE-luciferase reporter gene assay. Representative experiments are shown.
However, the maximal amount of H4Rs labeled by [3H]histamine (B\text{max}) decreased upon co-transfection of individual cDNAs coding for the H4R(390) and the H4R splice variants in a 1:1 cDNA ratio compared to cells transfected only with cDNA encoding the H4R(390). A decrease in B\text{max} of 30 ± 5% (n=3) was observed when the H4R(390) was co-expressed with the H4R(67). An even larger decrease of 55 ± 3% (n=3) was observed when co-expressed with the H4R(302) (Figure 4.5A, Table 4.2). The decrease in B\text{max} is directly related to the ratio of H4R(390) and H4R(302) cDNA that was transfected. Transfecting more H4R(302) cDNA results in a concomitant decrease of the B\text{max} value for [3H]histamine (Figure 4.5B). This decrease was not a result of saturation of the translation system, as co-expression of H1R, which does not physically interact with the H4R (234) had no negative influence on H4R expression (Figure 4.5C). We also tested a set of typical H4R ligands for their affinity in cells expressing the H4R(390) alone or when co-expressed with the H4R(302) and H4R(67). No difference in K\text{a} values for the selective H4R agonist 4-methylhistamine, the H4R inverse agonist thioperamide, the non-imidazole H4R neutral antagonist JNJ 7777120 and the non-imidazole H4R agonist clozapine (115,148) were observed (Figure 4.5C, Table 4.2). We further investigated the effect of co-expression of the H4R splice variants on the level of receptor signaling. We co-transfected H4R(390) in a 1:1 cDNA ratio with either the H4R(302) or H4R(67) and a CRE-luciferase reporter gene (Figure 4.5E). Both the co-expression of H4R(302) and H4R(67) resulted in a significant (p<0.05) decrease in pEC\text{50} of histamine (8.4 ± 0.1, n=4) compared to when H4R(390) is expressed alone (8.8 ± 0.1, n=6).

**Co-expression of the H4R(390) with the H4R splice variants results in reduced surface expression of the H4R(390).**

To determine the location of the H4R(390) upon co-expression with either of the two H4R isoforms a biotinylation-surface labeling assay was performed in combination with Western blot analysis using our previously described anti-H4R antibody (234) on HEK 293 cells either individually expressing the H4R(390) or co-expressing the H4R(390) with the H4R(302) or the H4R(67). In agreement with the ELISA results, the H4R(390) is expressed on the cell surface when expressed individually (Figure 4.6, lane 1). As reported previously (234), the H4R(390) is detected both as a monomer and
Chapter 4

as a dimeric receptor. However, upon co-expression of the H₄R₃⁹₀ with the H₄R₃⁰₂ or H₄R₆⁷ a clear reduction in intensity in anti-H₄R reactivity is observed in the cell surface fraction (Figure 4.6, lanes 4 and 7). This reduction in apparent H₄R surface expression is clearly dependent on the amount of splice variant co-transfected with the H₄R₃⁹₀. Increasing the amount of H₄R isoform 5-fold causes a stronger reduction in anti-H₄R reactivity (Figure 4.6, lanes 5 and 8). This reduction was not observed when co-transfecting identical amounts of GFP (Figure 4.6, lane 2).

**Figure 4.6. Reduced surface expression of H₄R₃⁹₀ when co-expressed with H₄R isoforms.** HEK 293 cells individually expressing H₄R₃⁹₀ or co-expressing H₄R₃⁹₀ with either GFP (control), H₄R₃⁰₂ or H₄R₆⁷ with cDNA ratios of 1:1 and 1:5, respectively. Intact cells were biotinylated for 15 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin, washed and homogenised. Biotinylated surface fractions were isolated by streptavidin chromatography, and analyzed by immunoblotting. Surface fractions: lane 1: H₄R₃⁹₀ alone; lane 2: H₄R₃⁹₀+ GFP (1:5 ratio); H₄R₃⁹₀+ H₄R₃⁰₂ (1:1 ratio); lane 3: H₄R₃⁹₀ alone; lane 4: H₄R₃⁹₀+ GFP; lane 5: H₄R₃⁹₀+ H₄R₃⁰₂ (1:5 ratio); lane 6: H₄R₃⁹₀ alone; lane 7: H₄R₃⁹₀ + H₄R₆⁷ (1:1 ratio); lane 8: H₄R₃⁹₀ + H₄R₆⁷ (1:5 ratio). All lanes were probed with anti-H₄R receptor antibody.

**Hetero-oligomerization between H₄R₃⁹₀ and H₄R splice variants.**
We hypothesized that hetero-oligomerization between the H₄R₃⁹₀ and the H₄R splice variants might be responsible for intracellular retention of the H₄R₃⁹₀ and the
observed decrease in $[^3H]$histamine binding to the co-expressed H4R(390). We therefore initially performed tr-FRET measurements to measure hetero-oligomerization. For this purpose, COS-7 cells co-expressing N-terminally HA-tagged histamine H4R(390) (HA-H4R(390)) and either FLAG-H4R(302) or FLAG-H4R(67) were incubated with Europium (Eu$^{3+}$) labelled anti-HA antibodies or a combination of the Eu$^{3+}$ labelled anti-HA and allophycocyanin (APC) labelled anti-FLAG antibodies.

Figure 4.7. Evaluation of hetero-oligomerization between the HA-tagged H4R(390) and FLAG-tagged H4R splice variants H4R(302) and H4R(67) by tr-FRET using co-expression of differentially epitope-tagged receptors. Transiently transfected COS-7 cells co-expressing the HA-H4R and the FLAG-H4R(302) or FLAG-H4R(67) in a 0.2:1 or 1:1 cDNA ratio were incubated for 2 hours with the Eu$^{3+}$-labelled anti-FLAG and APC-labelled anti-HA antibodies. As a controls tr-FRET was performed on COS-7 cells co-expressing the FLAG-H4R and triple HA-tagged S1P1R or HA-H4R(390). Data are normalized for the tr-FRET signal obtained from a mixture of COS-7 cells that individually expressing the indicated constructs. Data shown are average of at least three experiments each performed in triplicate.
The resultant data were normalized against tr-FRET signals obtained from COS-7 cells individually expressing the FLAG-tagged H₄R splice variants and the HA-H₄R(390)s that were mixed and exposed to the two antibodies. Upon co-expression of the HA-H₄R(390) in a 0.2:1 cDNA ratio with the FLAG-H₄R(302) or the FLAG-H₄R(67) isoforms a significant tr-FRET signal could be observed compared to the tr-FRET signal obtained from a mixture of cells individually expressing the two receptors (Figure 4.7).

Increasing the amount of HA-H₄R(390) co-transfected with the H₄R splice variant 5-fold to a 1:1 cDNA ratio, resulted in an further increase in tr-FRET signal especially for the H₄R(390)-H₄R(67) hetero-oligomer (Figure 4.7). As a control, we used COS-7 cells co-expressing the FLAG-H₄R(390) and triple HA-tagged sphingosine-1-phosphate (EDG1) receptor (3xHA-S₁P₁R). Although the S₁P₁R and the H₄R(390) are co-localized in a variety of cell types ²³⁵ and the S₁P₁R is known to form homo- and hetero-oligomers ²³⁶, no hetero-oligomerization occurred between these two receptors in COS-7 cells (Figure 4.7).

Additional support for hetero-oligomerization between H₄R(390) and the two H₄R splice variants arose from co-immunoprecipitation studies. HEK 293 cells co-expressing either the H₄R(390) with FLAG-H₄R(390), FLAG-H₄R(302) or FLAG-H₄R(67) were solubilized and immunoprecipitated with anti-FLAG Sepharose beads and subsequently immunoblotted using the anti-H₄R antibody. In all three cases the anti-H₄R antibody was able to detect the H₄R(390) (Figure 4.8, lane 2, 4 and 6). As the anti-H₄R antibody recognizes the C-terminal tail of the H₄R(390) the antibody can still react with the H₄R(302) but not with the H₄R(67). Western blot analysis on immunoprecipitated FLAG-H₄R(302) clearly shows the presence of the full length H₄R(390) as well as the shorter H₄R(302). Additionally, a broad high molecular weight species can be distinguished, most likely consisting of homo-dimeric H₄R(390) and FLAG-H₄R(302) and hetero-dimeric H₄R(390)-FLAG-H₄R(302) (Figure 4.8, lane 4). Interestingly, no monomeric H₄R(390) was detected in the immunoprecipitated FLAG-H₄R(67) sample (Figure 4.8, lane 6). As control, transfected HEK 293 cells were immunoprecipitated with Sepharose beads. In these samples no detectable H₄R was precipitated (Figure 4.8, lanes 1, 3 and 5).
Cloning of dominant negative H₄R splice variants

**Figure 4.8.** Immunoprecipitation evidence for H₄R₃⁹₀ interaction with hH₄R isoforms, H₄R₃⁰₂, and H₄R₆⁷. HEK 293 cells were co-transfected with equal amounts of H₄R₃⁹₀ and either FLAG-H₄R₃⁹₀, FLAG-H₄R₃⁰₂ or FLAG-H₄R₆⁷. Cells were harvested 40h post transfection, solubilised with 1% Triton-X100 and subjected to immunoprecipitation with anti-FLAG sepharose or sepharose control beads for 2h at 4°C. Following washing, bound material was collected using SDS-PAGE sample buffer and analyzed by immunoblotting. Immunoblots were probed with anti-H₄R antibody. Lane 1, 3 and 5 are control precipitations with sepharose beads; Lane 2, 4, and 6 are precipitations with anti-FLAG sepharose. Protein species identified were consistent with the dimeric and monomeric H₄R₃⁹₀ or H₄R₃⁰₂ species, respectively. The H₄R₆⁷ was not detected by this antibody.

**DISCUSSION**

The hH₄R, the latest member of the histamine receptor family, is expressed abundantly on hematopoietic cells and its role in eosinophil and mast cell functions points to a potential role in various allergic and inflammatory conditions. Within the histamine receptor family the H₄R shares highest
homology with the H₃R, up to 58% within the transmembrane (TM) domains. Moreover, the organization of the gene encoding the H₄R closely resembles that of the H₃R gene, as both genes consist of three exons and two introns. In recent years, a large number of alternatively spliced isoforms have been discovered for the H₃R. In our attempts to determine if alternative splice variants also exist for the H₄R, we describe here for the first time two isoforms of the human H₄R, which we cloned from cDNA from human CD34⁺ cord blood cells derived eosinophils and mast cells. The first splice variant, lacks part of TMII, TMIII and TMIV (H₄R₃02), while the second splice variant is a heavily truncated H₄R and contains only TMI and the first half of TMII (H₄R₆7).

Quantitative PCR showed that in different cell types mRNA encoding the H₄R₃90 the H₄R splice variants are differentially expressed. The H₄R splice variant mRNA was most abundant in eosinophils, which is in agreement with the fact that both isoforms were cloned from eosinophils. Interestingly, in pre-monocytes the expression of H₄R₆7 mRNA resembles that of the H₄R₃90. The lower abundance for mRNA of H₄R splice variants in other cell types, could be due to reduced mRNA stability compared to the mRNA of the full-length receptor and appears to be common for other GPCR splice variants as well. Yet, it is possible that in different cells or under different (patho) physiological conditions the H₄R splice variants are expressed more abundantly, resulting in the dominant negative effect seen in our recombinant cell systems. Evidence supporting this possibility has been reported recently for the H₃R isoforms in the rat brain. Future studies should address these important questions.

In March 2003 Merck registered a patent (WO 03/020907 A2) in which they describe the discovery of two splice variants of the hH₄R, H₄bR and H₄cR, cloned from human spleen cDNA. While the alternative splicing of the H₄R that leads to the formation of the H₄bR and the H₄R₆7 is identical, the H₄cR appears to result from alternatively splicing event after exon 2. The H₄cR is the result of the recognition of an alternative acceptor site in exon 3, resulting in a 33 amino acid deletion near TMIV. Interestingly, the reported sequences of both the H₄bR and H₄cR contain additional amino acids, which we have not observed in the identified H₄R₆7 or H₄R₃02 and can also not be explained based on the H₄R genomic
Cloning of dominant negative H4R splice variants

sequence. According to the patent (WO 03/020907 A2), the H4bR and H4cR are able to bind $[^3H]$histamine and to signal via several functional pathways, such as Ca$^{2+}$-mobilization, inhibition of cAMP production and the activation of mitogen activated protein kinase (MAPK). In our search for H4R isoforms we did not detect the H4bR and H4cR in immature dendritic cells (DC), differentially activated DCs (LPS or TNF$\alpha$/IL$\beta$1), mast cells, eosinophils or basophils. Using PCR, we constructed both the putative H4bR and H4cR isoforms, but in contrast to the findings of Merck, we were not able to detect any specific $[^3H]$histamine binding using these splice variants (data not shown).

Both the H4R$_{(302)}$ and H4R$_{(67)}$ lack TMIII, which harbors the key aspartate residue, D$_{94}$ (3.32), presumed to be crucial for histamine binding, as well as for the high affinity binding of other known orthosteric H4R ligands $^{34}$. As expected the two novel H4R splice variants were unable to bind the agonist radioligand $[^3H]$histamine or the non-imidazole antagonist radioligand $[^3H]$JNJ 7777120 $^{172}$. Although the two isoforms apparently are unable to bind ligands with high affinity, it was still possible that the two receptor isoform could signal constitutively or at high histamine concentrations. Previous studies have shown that the H4R$_{(390)}$ is constitutively active and can be further activated by H4R agonists $^{115,148}$. However, whereas PTX treatment or the inverse agonist thioperamide inhibit the constitutive activity of the H4R$_{(390)}$, no effect of thioperamide or PTX treatment was observed for both new H4R isoforms. Moreover, also high concentrations of histamine were unable to modulate H4R signal transduction in cells expressing either one of the two H4R isoforms. These data indicate that neither the H4R$_{(302)}$ nor H4R$_{(67)}$ alone are able to (constitutively) activate the Gi/o-coupled signaling pathway by themselves.

Since both the H4R$_{(302)}$ and H4R$_{(67)}$ isoforms are unable to signal and are expressed predominantly intracellular as determined by ELISA and Western blot experiments, we decided to explore the effects of these splice variants upon co-expression with the H4R$_{(390)}$. Dominant negative splice variants of GPCRs, influencing the functionality (e.g. signaling or trafficking) of the full-length receptor have been shown for a variety of GPCRs such as the histamine rH3R $^{204}$, the dopamine D3R $^{230}$, the gonadotropin-releasing hormone receptor GnRH $^{239}$ and the growth hormone receptor GHR $^{240}$. In all these cases, the reported dominant negative
effect of the receptor isoforms was presumably due to hetero-oligomerization between the full-length receptor and the receptor isoform. Upon co-expression of the $H_4R_{(390)}$ with the $H_4R_{(302)}$ or the $H_4R_{(67)}$ isoforms a clear decrease in $B_{\text{max}}$ for $[^3\text{H}]\text{histamine}$ was observed. However, the ligand binding properties of the remaining $[^3\text{H}]\text{histamine}$ binding sites were unaffected as the $K_D$ value for $[^3\text{H}]\text{histamine}$ as well as $K_i$ values of other $H_4R$ ligands did not change when the $H_4R_{(390)}$ is co-expressed with either of the $H_4R$ splice variants. As expected, the reduction in $H_4R$ expression induced by the $H_4R$ splice variants was paralleled by a decrease in the potency of histamine in the CRE-driven luciferase reporter gene assay. The apparent reduction in $B_{\text{max}}$ could be explained by reduced cell-surface expression of $H_4R_{(390)}$. We have recently shown that three non-functional $rH_3R$ splice variants have a dominant negative effect on the cell-surface expression of full-length $H_3Rs$, presumably by $rH_3$ isoform hetero-dimerization \(^{204}\). We have previously shown by various methods that also the $H_4R_{(390)}$ can readily form homo-oligomers \(^{234}\). In the present study \textit{time-resolved} FRET as well as co-immunoprecipitation experiments revealed that the $H_4R_{(390)}$ can also form cell-surface hetero-oligomers with both the $H_1R_{(302)}$ or the $H_4R_{(67)}$ isoforms. Similar to the our previous reported on $H_4R$ homo-dimers, we find that the homo-dimers and hetero-dimers are (partially) insensitive to SDS and DTT, suggesting that these dimers may interact via strong hydrophobic interactions, as has been reported for e.g. the $H_3R$ receptor \(^{204}\) and the $\mu$-opioid receptor-$\alpha_2\alpha$-adrenergic receptor hetero-oligomer \(^{241}\). Co-expression of the $H_4R$ splice variants resulted in a clear reduction in the surface expression of the $H_4R_{(390)}$ as determined in a surface labeling assay. The reduction in $H_4R$ binding and surface expression is not merely caused by saturation of the expression system, as both co-transfection of $H_1R$ and GFP did not have any negative influence on the expression of $H_4R$. These findings resemble our previous findings with the $rH_3$DEF isoforms, which trap all full-length $rH_3R$ intracellularly \(^{204}\). However, the herein described $H_4R$ splice variants resemble more the $D_3nfR$ than the $rH_3R$ isoforms, which seem to be expressed exclusively intracellularly \(^{204}\). As with $H_4R$ isoforms, the $D_3nfR$ is non-functional, expressed at the cell surface to some extent and reduces the binding of radioligand to the $D_3R$ through hetero-oligomerization \(^{230}\).
Hetero-oligomerization between the H$_4$R$_{(390)}$ and the H$_4$R$_{(67)}$ appears to have a less pronounced effect on the cell surface location of the H$_4$R$_{(390)}$ compared to the H$_4$R$_{(302)}$ (reduction in B$_{max}$ 70% vs. 45% at similar cDNA ratios). However, tr-FRET assays show that the H$_4$R$_{(67)}$ may form hetero-oligomers with the H$_4$R$_{(390)}$ more readily. Moreover, co-immunoprecipitation data of the H$_4$R$_{(67)}$ with the H$_4$R$_{(390)}$ indicate the assembly of predominantly high molecular weight species. One might speculate that the small H$_4$R$_{(67)}$ is able to form higher oligomeric complexes (trimers, tetramers) more readily than the larger H$_4$R$_{(302)}$ isoform with H$_4$R$_{(390)}$, but has less cytoplasmic (ER) retention properties. More detailed studies (e.g. employing triple BRET/FRET assays using bimolecular fluorescence complementation) are required to further investigate this hypothesis.

The observed hetero-oligomerization between the H$_4$R$_{(390)}$ and either of the two H$_4$R splice variants also offers insight into the domains involved in H$_4$R oligomerization. In general, family A GPCRs are thought to oligomerize by interaction of TM domains, either via the formation of contact-dimers or by domain-swapping. When focusing on aminergic receptors, several TM domains have been reported to be involved in the mechanism of oligomerization. For example, the dopamine D2 receptor, the involvement of TMIV in the oligomer interface has been clearly demonstrated. However, our data from the heavily truncated H$_4$R$_{(67)}$ reveals that for the H$_4$R TM IV is not crucial for the formation of H$_4$R dimers, whereas TMII must be involved. This is similar to the $\alpha_{1B}$-adrenergic receptor for which TMII is important for homo-oligomerization. Since TMII of H$_4$R$_{(67)}$ is partly truncated and has so far not been directly correlated with involvement in GPCR oligomerization, we propose TMII as an important domain involved in H$_4$R oligomerization.

In summary, we have cloned and characterized two new splice variants of the human H$_4$R from CD34+ cord blood derived eosinophils. Although both H$_4$R splice variants are non-functional and mainly localized intracellularly, they can hetero-oligomerize with the H$_4$R$_{(390)}$, thereby reducing the ability of the H$_4$R$_{(390)}$ to reach the cell surface and functionally bind histamine. As mRNA of these splice variants is differentially expressed in different cell types, we propose that under certain
conditions, these newly discovered H$_4$R splice variants may have a role in the regulation of H$_4$R$_{(390)}$ function.

**MATERIALS AND METHODS**

**Materials.** Reagents for tr-FRET were from Cis Bio International (Bagnols-sur-Cèze Cedex, France). Chloroquine diphosphate, DEAE-dextran (chloride form), histamine(2-[4-imidazolyl]ethylamine hydrochloride), 3,3',5,5'tetramethyl benzidine (TMB) monoclonal mouse M2 anti-FLAG (DYKDDDDK), N,N-dimethylformamide, pertussis toxin, clozapine and polyethyleneimine was purchased from Sigma (St.Louis, MO). High affinity rat anti-HA (YPYDVPDYA) was from Roche Applied Science (Basel, Switzerland). Goat anti-mouse IgG (H+L) HR was obtained from Bio-Rad (Hercules, USA), polyclonal rabbit anti-Rat IgG HR was from Dakocytomation (Glostrup, Denmark). Calf serum was purchased from Integro BV (Dieren, The Netherlands). Polyethyleneimine (linear, MW~25.000) was from Polysciences Inc (Warrington, PA, USA). D-luciferin was from Duchefa (Haarlem, The Netherlands). Cell culture media, penicillin, streptomycin and TRIzol were obtained from Invitrogen (Breda, The Netherlands). Cell culture plastics were from Greiner Bio-one (Wemmel, Belgium). Tris was from AppliChem (Darmstadt, Germany). [${}^3$H]histamine (18.10 Ci/mmol) were purchased from Perkin-Elmer Life Science, Inc. (Boston, MA). Oligonucleotides were purchased from Isogen Bioscience (Maarsen, The Netherlands). PfuTurbo® DNA polymerase was purchased from Stratagene (La Jolla, USA). Restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). The human H$_4$R$_{(390)}$ cDNA in pcDNA3.1 was purchased from the Guthrie cDNA resource center (Sayre, PA). The hH$_4$R was subcloned into the pcDEF$_3$ using BamHI/XbaI sites. Thioperamide fumarate, clobenpropit dihydrochloride, 4-methylhistamine and JNJ 7777120, were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam. Gifts of expression vector pcDEF$_3$ (Dr. J Langer) and [${}^3$H]JNJ 7777120 (Dr. R. Thurmond) are greatly acknowledged.

**Cloning and sequence analysis of the hH$_4$R$_{(302)}$ and H$_4$R$_{(67)}$ splice variants cDNAs.**

In order to identify and isolate H$_4$R splice variants, CD34+ cells were isolated from cord blood mononuclear cells and were cultured in 100 ng/ml stem cell factor (SCF) and 80 ng/ml interleukin 6 (IL6) for 8-10 weeks, in 5 ng/ml IL3 and 1 ng/ml transforming growth factor-β for 3-4 weeks or in 2 ng/ml IL3 and 5 ng/ml IL5 for 4-5 weeks, to differentiate the CD34+ cells to mast cells, basophils or eosinophils, respectively $^{286-288}$. Peripheral blood mononuclear cells were cultured in 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml IL4 to create immature dendritic cells, which were subsequently activated with either lipopolysaccharide (LPS) or a combination of tumor necrosis factor α and IL1β $^{150,249}$. Total RNA was isolated using the nucleo spin RNA II purification kit (Clontech Laboratories, Palo Alto, USA) and cDNA was obtained by random primed cDNA synthesis. A set of (nested) sense and anti-sense oligonucleotide primers, spanning the intron/exon junctions of the H$_4$R gene sequence, were used in order to identify potential H$_4$R splice variants. Use of the following sense primers 5'-TGTGATTGCCAGATCTATAA-3', 5'-ATGC CAGATACTAATAGC-3', 5'-
Cloning of dominant negative H4R splice variants 

TCCTTTGTGGTGATCTCC-3' and anti-sense primers 5'-TGTGATGGCAAGGATGTACC-3', 5'-TTAAAGAAGATCTGCCGC-3', 5'-CTTCTAAGAGATACTGCC-3' resulted in the amplification of at least two different products. The generated PCR fragments were cloned in pCRII-TOPO (Invitrogen) and the cDNAs were sequenced on both DNA strands. The sequence of the identified hH4R(302) and hH4R(67) have been deposited in the GenBank® database (accession number DQ835186 and DQ835187, respectively). The cloned cDNAs were transferred to a mammalian expression vector (pcDEF3), either untagged or tagged N-terminally with a FLAG-epitope (DYKDDDDK).

Cell culture and transfection. COS-7 and HEK 293T cells were maintained at 37°C humidified in 5% CO2/95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 50 IU/ml of penicillin and 50 μg/ml streptomycin and grown in 100 mm dishes. COS-7 Cells were transiently transfected using the DEAE-dextran method as previously described 85. HEK 293T cells were transfected using the polyethyleneimine (PEI) transfection method adapted from Durocher et al 250. Briefly, 5 μg DNA was diluted in 500 μl 0.9% NaCl, and 10 μl of a 1 mg/ml PEI (linear MW~25.000) was added. Following ten minutes of incubation the mixture was added to the HEK 293T cells. 24 hours after transfection, cells were transferred into 96 well plates. In all experiments the total amount of DNA transfected was maintained constant by addition of the empty expression vector pcDEF3.

Culture and differentiation of HL-60 cells. HL-60 suspension cells were maintained at 37°C humidified in 5% CO2/95% air atmosphere in RPMI 1640 + L-Glutamine supplemented with 10% (v/v) fetal bovine serum and antibiotics. Cells were differentiated to granulocytes and eosinophils. HL60 cells were incubated for 5 days with 0.65% (v/v) N, N-dimethylformamide to differentiate the cells to granulocytes. Eosinophilic differentiation was performed by culturing HL-60 cells in the presence of 0.5 mM butyric acid for 48h. IL-5 was added to a concentration of 10 ng/ml, and the cells were further cultured for 72h 148. Total RNA was isolated using TRIzol according to manufacturer’s protocol. cDNA was synthesized using Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was subsequently used in Q-PCR

Radioligand binding studies. Cells used for radioligand binding studies were harvested 48h post transfection and homogenized in ice cold hH4R binding buffer (50 mM Tris, pH 7.4). For saturation isotherms cell membrane homogenates were incubated at 37°C for 60 minutes with 0-150 nM [3H]histamine or 0-30 nM [3H]JNJ 7777120 172 in a total assay volume of 200 μl. For competition binding assays the cell homogenates were incubated at 37°C for 60 minutes with 0.1-10.000 nM ligand in the presence of ~15 nM [3H]histamine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice cold hH4R binding buffer. The bound radioactivity was separated by filtration through GF/C filterplates (Perkin Elmer) that had been treated with 0.3% PEI. Filters were washed four times with ice cold hH4R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.
**CRE-luciferase reporter gene assay.** HEK 293T cells transiently co-transfected with 2.5 μg pTNLC-121 and 5 μg pcDEF-3-H4R, pcDEF3-H4R(302), pcDEF3-H4R(67) were seeded in 96-well white-plates in culture medium and after 42h incubated with histamine or thioperamide and 0.5 μM forskolin for 6h. After 48h, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μl/well luciferase assay reagent [0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM MgCl₂, 0.78 μM Na₂H₂P₂O₇, 38.9 mM Tris, pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 μM dithiothreitol]. After 30 min, luminescence was measured for 3 s/well in a Victor² 1420 Multilabel Counter (PerkinElmer, Boston, USA).

**Enzyme linked immunosorbent assay (ELISA).** 48h after transfection, receptor expression in COS-7 cells was measured using an ELISA assay as previously described. To detect HA (hemagglutinin)-epitope tagged receptors a rat anti-HA antibody was used as primary antibody, and a rabbit anti-rat–horseradish peroxidase conjugate as secondary antibody. To detect FLAG-epitope tagged receptors a mouse anti-FLAG antibody was used as primary antibody and a goat anti-mouse–horseradish peroxidase conjugate as secondary antibody. The 3', 3', 5, 5'-tetramethylbenzidine liquid substrate system for ELISA was used as substrate and the optical density was measured at 450 nm using a Victor² 1420 Multilabel Counter.

**Quantitative PCR.** The integrity and quality of isolated total RNA was determined by agarose gelelectrophoresis and concentration was determined spectrophotometrically. Next, 1μg of total RNA was DNase treated at 37°C for 15 min (RQ1 DNase, Promega), and the reaction terminated at 60°C for 10 min. The treated RNA was directly subjected to 1st strand cDNA synthesis (Iscript, Bio-Rad) in 20 μl. The synthesized cDNA was diluted to 500 μl with distilled water and then used for Q-PCR (MyIQ, Bio-Rad) in a 20 μl reaction (2x SYBR buffer 10 μl, 25 pM primers, and cDNA 5.0 μl). The reaction was stopped after the 40th PCR cycle was completed. Primers of H4R(390) and two splice variants H4R(302) and H4R(67) were designed using Beacon designer software (Bio-Rad). A sense primer was designed that recognizes both H4R(390) and the H4R splice variants 5'-TGCTAGGAAATGCTTTGGTC-3'. Isoform specificity was obtained by designing anti-sense primers that are only able to bind to the cDNA of either the H4R(390), 5'-GCCTGTTAAGGATCTCGACA-3', the H4R(302) 5'-TCTAGACTGTGAACCCACTAA-3' or the H4R(67) 5'-TCTAGACTCTGAAACCACA-3'. Specificity of primers was evaluated by checking dissociation curve and amplification slope. For data analysis, the nuclear ribosome protein 11 (rps11, NM001015) was used as reference to H4R expression. Expression level was analyzed based on 2-ΔΔCt value method.

**tr-FRET assay.** tr-FRET assays were performed using a combination of both Eu³⁺-labeled and allophycocyanin-labeled anti-FLAG and anti-HA antibodies as described previously. Briefly, tr-FRET was assessed in 5 x 10⁵ intact COS-7 cells, transiently expressing the appropriate HA and FLAG tagged receptors. Cells were incubated in PBS containing 50% FCS (v/v), 0.8 nM of Eu³⁺-labelled antibody, and 8 nM of allophycocyanin-labelled antibody for two hours at room temperature. After two wash steps the
cells were resuspended in 50 µl PBS and transferred to a 384-microwell plate. Energy transfer was measured by exciting the Eu³⁺ at 320 nm and monitoring the allophycocyanin emission for 500 µs at 665 nm using a Novostar (BMG Labtechnologies, Offenberg, Germany) configured for τ-fluorescence after a 100 µs delay.

**Immunoprecipitation of H₄R isoforms.** HEK 293 cells were transfected with H₄R(390) with or without FLAG-H₄R(302) or FLAG-H₄R(67). Cell samples were solubilised with 1% Triton-X100 in 50 mM Tris-HCl pH 7.4 and incubated with 50 µl 50% (w/v) slurry of anti-FLAG Sepharose beads (Sigma, UK) for 2 hrs at 4°C. Following incubation, beads were centrifuged at 9000rpm at 4°C for 5 min. The supernatant was retained (unbound fraction) and the beads washed in 50 mM Tris-HCl pH 7.4/Triton X100 and bound fraction isolated by elution in SDS PAGE Sample buffer/50mM DTT at 50°C for 30 min. Supernatant following centrifugation at 9000 rpm was retained (bound fraction) for analysis.

**Surface biotinylation assay.** HEK 293 cells were transfected with clones encoding individual H₄R isoforms or co-transfected with H₄R(390) with variable amounts of green fluorescent protein (GFP, control), H₄R(302) or H₄R(67) isoforms (1:1 or 1:5). The medium was carefully removed 24-40 hours post transfection and cells were washed with ice-cold PBS/4 % (w/v) sucrose (3x1 ml per 35mm dish). The cells were then incubated for 15 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin in ice-cold PBS/4 % (w/v) sucrose (0.5 ml/dish) with gentle shaking. Subsequently, cells were washed once with ice- cold PBS/4 % (w/v) sucrose (1ml/dish), and incubated for 10 min at 4°C with quenching buffer (192mM glycine, 4% (w/v) sucrose in 50mM Tris-HCl pH 8.0 ) (0.5 ml/dish). Cells were scraped into PBS/I4 % (w/v) sucrose and spun at 6000 rpm for 2 min. This wash procedure was repeated on a further occasion and the cells were homogenised in 1% (w/v) SDS in lysis buffer (50mM Tris-HCl, pH 8.0/ 2 mM EDTA/1% Triton-X100 The samples were then diluted five-fold in lysis buffer (without SDS), and incubated with 20 µl 50% (w/v) slurry of streptavidin beads for 2 hrs at 4°C. Following incubation, beads were centrifuged at 9000rpm at 4°C for 5 min. The supernatant (intracellular fraction) was retained and the beads washed in lysis buffer. The bound fraction was isolated by elution in SDS PAGE Sample buffer/50mM DTT at 50°C for 30 min. Supernatants following centrifugation at 9000 rpm were retained (surface fraction) for analysis.

**Immunoblotting.** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 6 % or 7.5 % polyacrylamide slab gels under reducing conditions. Samples of HEK 293 cells (20-50 µg protein) were prepared using a chloroform/methanol method of protein precipitation, and immunoblotting was performed as previously described 203,204,234. Immunoblots were probed with anti-H₄R 374-390 antibody 234 at a concentration of 0.5 µg /ml. Blots containing FLAG- or HA-tagged receptors were probed with primary antibodies, mouse anti-FLAG (1.5 µg/ml) or rat anti-HA (1 µg/ml), respectively. Horseradish peroxidase conjugated goat anti-mouse or anti-rat antibodies (1:2000-5000) were used as secondary antibodies (Little Chalfont, Buckinghamshire, England).
Analytical methods. All radioligand binding data were evaluated by a non-linear least squares curve fitting program using Graphpad Prism® (Graphpad Software Inc, San Diego, CA). Protein concentrations were determined according to Bradford, using BSA as standard. All data are represented as mean ± S.E.M. from at least three independent experiments in triplicate. Statistical significance was determined by an ANOVA-tukey test (p<0.05 was considered statistically significant).
CHAPTER 5


Van Rijn RM, Bakker RA, Leurs R
Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
ABSTRACT

The oligomeric potential of G protein coupled receptors (GPCRs) has become generally accepted over the last years. Whereas for class C GPCRs, oligomerization has been shown to be pivotal for receptor function, receptor oligomerization of GPCRs belonging to other classes has thus far not been shown to be essential for receptor function. Whereas atomic force microscopy of native rhodopsin showed them to be arranged in rows of dimers it is still unclear if all GPCRs oligomerize in a similar fashion; whether the interaction points are the same, and what the fate is of oligomers upon ligand stimulation, desensitization or receptor internalization. While focusing primarily on GPCRs belonging to the class A subfamily of aminergic receptors, we provide a detailed summary of recently published data on GPCR oligomerization in order to answer some of the questions of when, where, how and why these receptors oligomerize. Finally, we try to identify gaps in current knowledge with suggestions for future investigations regarding oligomerization of aminergic receptors or GPCRs in general.

What is receptor oligomerization?

Originally G-protein-coupled receptors (GPCRs) were considered to function as monomers i.e. 7 trans-membrane proteins comprised of a single peptide chain. However, compelling evidence in recent years has shifted this believe to the current hypothesis where two or more GPCRs may associate to form oligomeric polypeptides. This shift began a couple of decades ago with observations of complex binding kinetics \(^{253,254}\) and of higher molecular weight bands in both Western blots and photo-affinity labeling experiments, which provided indications for the existence of GPCR oligomers \(^{255-257}\). More recent studies using biophysical approaches in living cells have generated more compelling evidence to support this new hypothesis.

In fact, oligomerization of proteins is a very common phenomenon \(^{258,259}\) and has been shown to occur between receptors within each of the three major classes of
mammalian GPCRs; class A (rhodopsin, olfactory, opioid, chemokine), class B (calcitonin, secretin, vasoactive intestinal peptide) and class C (GABA, metabotropic, calcium sensing, taste). While for the class C family of GPCRs a certain consensus for oligomerization has been observed e.g. a carboxyl terminal coiled coil interaction and disulfide bridges of the “venus flytrap” binding domains, oligomerization of members of class A family of GPCRs seem to follow less specific rules. A subfamily of the class A GPCRs are the aminergic receptors. Besides sharing the hallmarks of class A GPCRs e.g. aspartate residues within transmembrane (TM) domain II and TM III, tryptophanes in TM IV and TM VI, a NPXXY motif in TM VII, a feature of aminergic receptors is that the ligand binding domain is localized within the TM domain. The focus of this review is to provide a detailed summary of research regarding oligomerization of GPCRs belonging to the subfamily of aminergic receptors with an emphasis on when, where and how these receptors form oligomers. Moreover, the partner specificity for hetero-oligomerization and the functional role of these oligomers will be discussed, as well as future prospects in the study of GPCR oligomerization.

**Methods for studying GPCR oligomerization.**

A diverse array of techniques has been employed to study GPCR oligomerization. Early indications for GPCR oligomerization came from Western blot and photo-affinity labeling studies in which the presence of high molecular bands were detected in addition to bands corresponding to the expected weight of monomeric receptors. Maggio and co-workers pioneered the study of receptor oligomers utilizing an approach based on chimeric receptors. Co-immunoprecipitation emerged in the late 90’s as a valuable technique to study GPCR oligomerization. Performing affinity chromatography prior to co-immunoprecipitation, allows the study of the interaction between three differentially tagged receptors. Although not commonly employed, the yeast two hybrid system can be a useful tool in studying the putative oligomer interface and discovering novel oligomeric partners.
With the arrival of biophysical techniques such as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) a vast amount of evidence supporting GPCR oligomerization has been produced. In brief, both assays are based on the principle that resonance energy originating from a donor can excite a fluorescent acceptor if in close enough proximity, usual within 100Å. The difference between FRET and BRET lies with the donor used. While FRET employs a fluorescent probe, BRET donor energy is emitted by enzymatic conversion of a substrate (usually coelenterazine) into light/energy (for reviews on the advantages and disadvantages of BRET and FRET see 211,213,271). Frequently, different types of green fluorescent protein (GFP) are used e.g. for FRET CFP (cyan) and YFP (yellow), or for BRET renilla luciferase (Rluc) and YFP. However, also fluorescently labeled ligands can function as energy donor 272. Aminergic receptors predominantly bind small, non peptidergic ligands, which are more difficult to be conjugated with bulky fluorescent groups without affecting the ligands affinity and specificity too much. However, several fluorescent ligands have been developed 273,274, which would facilitate the use of such a FRET based assay for studying oligomerization of aminergic GPCRs.

Over the years several adaptations to these RET assays have emerged (Figure 5.1). The introduction of BRET^2 provides improved signal resolution over BRET 275, whereas the introduction time-resolved FRET (tr-FRET) 276 facilitated the study of oligomers at the cell surface. An additional advantage of tr-FRET is that it only requires recombination of small N-terminal epitope tags plus the use of differentially labeled antibodies. The receptor is not required to be fused with large fluorescent proteins, which have a larger potential of changing receptor pharmacology compared to the epitope tags. Another adaptation to FRET is known as photobleaching FRET (pbFRET) or fluorescence recovery after photobleaching (FRAP). This technique allows for the study of lateral movement of receptors and receptor dimers on the cell surface by photobleaching a specific area of the cell surface and measure recovery of the fluorescent signal 277. Very recently, a new derivation of BRET, extended BRET (eBRET), has been described, which makes use of a new derivative of the coelenterazine substrate named EnduRen™ 278.
Unlike coelenterazine or DeepBlueC™ (BRET substrate) this substrate is stable at 37°C for prolonged periods of time, allowing for extended real-time BRET experiments. This method can potentially be used to follow a receptor homo-dimer or hetero-dimer in time. A technique that can be very useful in the study of oligomerization between more than two different proteins is bimolecular fluorescence complementation, a technique that combines BRET with PCA (protein fragment complementation assay). In this case YFP is split into two domains which can be linked to two different partners. Only if these two partners form hetero-oligomers a functional YFP is created that can function as acceptor in the BRET assay. In principal, the Renilla luciferase (Rluc) can also be split into two domains allowing for the study of four interacting partners. Another technique to measure interactions between multiple proteins is the three-fluorophore FRET. For this technique receptors are labeled with one of three different fluorophores. In a recent study the oligomerization state of the α1b-AR was investigated by fusing the receptors with eCFP, eYFP or dsRed. Resonance energy of eCFP is unable to directly excite dsRed, but eCFP can indirectly excite dsRed via excitation of eYFP, which resonance energy is capable of directly exciting dsRed.

The use of atomic force microscopy was successfully employed to reveal rhodopsin formed rows of dimers, but so far no other GPCR dimers have been studied using this technique. In 2005, Hoffmann and co-workers described a FLAsH based FRET assay, in which they employed a small, membrane-permeant fluorescein derivative with two arsen-(III) substituents (Fluorescein arsenical hairpin binder; FLAsH). When bound to a specific tetracysteine region (CCXXCC) of a GPCR, the FLAsH molecule could then function as an acceptor, whereas a GPCR fused to cyan fluorescent protein (CFP) functioned as a donor. The described FLAsH based FRET was employed to study intramolecular changes during receptor activation. However, the introduction of additional fluorescent groups; ChoXAsH and ReAsH, may facilitate the study of GPCR oligomerization using a FRET assay that requires neither large fluorescent proteins nor large conjugated antibodies. Moreover, the FLAsH based FRET approach may be combined with the use of fluorescent ligands.
Chapter 5

Figure 5.1. Overview of several biophysical techniques that are used to study GPCR oligomerization. From left to right the depicted techniques are bioluminescence resonance energy transfer (BRET), time-resolved fluorescence resonance energy transfer (tr-FRET), (three-fluorophore) FRET and bimolecular fluorescence complementation (BiFC). In all cases light from the donor can only excite the acceptor if the two are in close proximity (generally >100Å) of each other. In BRET the donor light is produced by enzymatic oxidation of coelenterazine, while for tr-FRET and FRET the donor has to be excited by an external light source. Abbreviations: Rluc, Renilla luciferase; YFP, yellow fluorescent protein; Eu³⁺, europium; APC, allophycocyanin; CFP, cyan fluorescent protein.

When and Where are oligomers formed?

It has been firmly established for the class C metabotropic GABA₉ receptors ⁴¹⁻⁴⁴, ²⁸⁴ as well as for the class B calcitonin receptors ²³³ oligomerization already occurs within the endoplasmatic reticulum (ER), prior to transport of the receptors to the cell membrane. In class A, a naturally occurring mutant of the CCR5 chemokine receptor is able to retain the wild-type CCR5 receptor intracellularly ²⁶⁹. Such dominant negative effects have been reported for other mutants of other receptor
and for receptor isoforms of aminergic GPCRs as well \(^{204,285,286}\). In contrast, expression of receptors that by themselves are retained intracellularly can be chaperoned to the cell surface via oligomerization. \(^{217,287}\). Hence, it appears class A hetero-oligomers are formed before or during translocation from ER/golgi to the plasma membrane. Indeed, measuring BRET or FRET signals in cell fractions collected from sucrose gradient, used to separate cell surface proteins from intracellular material, homo-oligomers could already be detected in light weight fractions (corresponding to ER/Golgi compartments) for the \(\beta_2\)-AR \(^{285}\), \(\alpha_{1A}\)-AR \(^{218,288}\) as well as hetero-oligomers between the D\(_1\)R and D\(_2\)R \(^{289}\). Real-time FRET and pbFRET studies on the biogenesis of 5-HT\(_{2C}\)-oligomerization convincingly revealed the presence of oligomers in the ER, and tracked them during transport to the Golgi and the plasma membrane \(^{290}\).

**Which receptors oligomerize and With Whom?**

Only a fraction of the total population of GPCRs has been studied in regard to oligomerization \(^{207}\). So far, with the single exception of the N-formyl peptide receptor \(^{291}\), all GPCRs, including the majority of aminergic receptors that have been studied were able to form homo-oligomers (Table 5.1). However, aminergic GPCRs appear to be more selective with respect to hetero-oligomerization. Often receptors sharing high homology, are able to hetero-oligomerize e.g \(\beta_2\)-AR-\(\beta_3\)-AR \(^{62}\), \(\alpha_{1B}\)-AR-\(\alpha_{1D}\)-AR \(^{217}\) 5-HT\(_{1B}\)-5HT\(_{1D}\) \(^{292}\). This is no rule of thumb; e.g. no interaction between \(\alpha_{1D}\)-AR and \(\alpha_{1A}\)-AR was detected \(^{217,293}\). Probably, if two receptors share high homology, the homo-oligomerization interface is identical to the hetero-oligomerization interface. But aminergic GPCRs hetero-oligomerization between different families of aminergic GPCRs (\(\beta_2\)-AR-5-HT\(_{4D}\) \(^{294}\), non-aminergic receptors belonging to class A (\(\beta_2\)-AR-DOR\(^{65}\), D\(_1\)R-A\(_3\)R \(^{295}\), D\(_2\)R-SSTR\(^{5}\))\(^ {272}\), ligand-gated channels; D\(_1\)R-NMDA \(^{296}\) and D\(_5\)R-GABA\(_A\) \(^{297}\) can also occur. It would be safe to state that in these cases the two partners do not have identical oligomerization interfaces. Thus different interfaces and interactions are required. Indeed, while the homo-oligomerization interface of the D\(_2\)R has been shown to involve
transmembrane domain IV (TM IV) hetero-oligomerization with the adenosine A$_{2a}$ receptor seems to involve an arginine-rich region in intracellular loop 2 (IL2), which can interact with a positively charged phosphorylated serine or a double aspartate “DD” region of the A$_{2a}$ receptor.

Still, one has to be careful when interpreting both homo- and hetero-oligomeric potential of receptors. In several cases hetero-oligomers between receptors can only be detected at very high, non-physiological expression levels. BRET saturation curves are very useful to determine the relative propensity of receptors to form hetero-oligomers, i.e. to establish if detected hetero-oligomers are physiologically relevant. For this to be true the different receptors also need to be actually co-localized within the same tissue.

Table 5.1. Overview of oligomerization of GPCRs belonging to the class A, subfamily of aminergic receptors. Represented are whether the receptors (histaminergic, adrenergic, dopaminergic, muscarinergic and serotonergic) have been reported to form homo- or hetero-oligomers. Also the specificity of hetero-oligomerization is depicted by listing receptors that have been reported not being able to form hetero-oligomers with the relevant receptor. Abbreviations: S1P1R, sphingosine 1 phosphate receptor; M71, M71 olfactory receptor; AT, angiotensin; TRHR, thyrotropin receptor; CCR5, chemokine receptor; CCKR cholecystokinin receptor; V$_1$R, vasopressin receptor; NE, no effect.

<table>
<thead>
<tr>
<th>Type</th>
<th>Receptor</th>
<th>Homo-oligomerization</th>
<th>Hetero-oligomerization</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>H1R</td>
<td>α1b-AR 245</td>
<td>α1p-AR, H4R 234</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H2R</td>
<td>α1d-AR 245</td>
<td>α1p-AR, H4R 234</td>
<td></td>
</tr>
<tr>
<td>H3R</td>
<td>α1d-AR</td>
<td>217</td>
<td>α1p-AR, H4R 234</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H1R</td>
<td>α1p-AR, H4R 234</td>
<td></td>
<td>H1R 234</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>α1b-AR</td>
<td>218,248</td>
<td>α1b-AR 218, DOR 258</td>
<td>M71, α1b-AR 217, 237</td>
</tr>
<tr>
<td></td>
<td>α1a-AR</td>
<td>205,218,245</td>
<td>α1b-AR 218, H1R 205,245, CCR5</td>
<td>M71, CCR5 218</td>
</tr>
<tr>
<td></td>
<td>α2A-AR</td>
<td>303</td>
<td>MOR 211, β2-AR 304</td>
<td>M71, α1p-AR 217</td>
</tr>
<tr>
<td></td>
<td>α2B-AR</td>
<td>286</td>
<td>β1-AR 286</td>
<td>M71, α1p-AR 217</td>
</tr>
<tr>
<td></td>
<td>α2C-AR</td>
<td>305</td>
<td></td>
<td>M71, α1p-AR 217</td>
</tr>
<tr>
<td></td>
<td>β3-AR</td>
<td>316</td>
<td>β3-AR 316</td>
<td>M71, α1p-AR 217</td>
</tr>
<tr>
<td></td>
<td>α1b-AR</td>
<td>242,248,249,300,301,311</td>
<td></td>
<td>TRHR 219, CCR5 214, CCKR 311</td>
</tr>
<tr>
<td></td>
<td>α1d-AR</td>
<td>217,205,245</td>
<td>α1b-AR 217, β2-AR 62, DOR 65, KOR 65</td>
<td>TRHR 219, CCR5 214, CCKR 311</td>
</tr>
<tr>
<td></td>
<td>β2-AR</td>
<td>316</td>
<td>β2-AR 316</td>
<td>TRHR 219, CCR5 214, CCKR 311</td>
</tr>
</tbody>
</table>

126
Table 5.1. Overview of oligomerization of GPCRs belonging to the class A, subfamily of aminergic receptors. Represented are whether the receptors (histaminergic, adrenergic, dopaminergic, muscarinic and serotonergic) have been reported to form homo- or hetero-oligomers. Also the specificity of hetero-oligomerization is depicted by listing receptors that have been reported not being able to form hetero-oligomers with the relevant receptor. Abbreviations: S1P1R, sphingosine 1 phosphate receptor; M71, M71 olfactory receptor; AT, angiotensin; TRHR, thyrotropin receptor; CCR5, chemokine receptor; CCKR cholecystokinin receptor; V1R, vasopressin receptor; NE, no effect.

<table>
<thead>
<tr>
<th>Type</th>
<th>Receptor</th>
<th>Homo-oligomerization</th>
<th>Hetero-oligomerization</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>D1R</td>
<td>299,318</td>
<td>A1R 255, NMDA receptor 296, D2R 299,319,320</td>
<td>α1a-AR 217, A3aR 321</td>
</tr>
<tr>
<td></td>
<td>D2LR</td>
<td>244,295,299,322-325</td>
<td>A2AR 255, NMDA receptor 295, D2R 299,319,320</td>
<td>α1a-AR 217, A3aR 321</td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>299,331,332</td>
<td>D1R 232, A2AR 294,321,326, D2R 289,319,320, S-</td>
<td>β2-AR 317</td>
</tr>
<tr>
<td></td>
<td>D4R</td>
<td>299,331,332</td>
<td>D2R 232, A2AR 294,321,326, D1R 289,319,320, S-</td>
<td>β2-AR 317</td>
</tr>
<tr>
<td>Muscarine</td>
<td>M1R</td>
<td>254</td>
<td>M2R 224</td>
<td>α1a-A 217, Smoothened 334</td>
</tr>
<tr>
<td></td>
<td>M2R</td>
<td>234,254,294,334,387</td>
<td>M2R 224</td>
<td>α1a-A 217, Smoothened 334</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT1BR</td>
<td>216,264,292,323,342</td>
<td>5-HT1BR 216, 5-HT1DR 216</td>
<td>α1a-AR 217, D1R 321</td>
</tr>
<tr>
<td></td>
<td>5-HT1DR</td>
<td>216,254,292,323,342</td>
<td>5-HT1BR 216, 5-HT1DR 216</td>
<td>α1a-AR 217, D1R 321</td>
</tr>
<tr>
<td></td>
<td>5-HT2CR</td>
<td>48,341</td>
<td>5-HT2CR 48,341</td>
<td>CCR5 200</td>
</tr>
<tr>
<td></td>
<td>5-HT3R</td>
<td>294</td>
<td>β2-AR 294, 5-HT4DR 294</td>
<td></td>
</tr>
</tbody>
</table>

How are oligomers formed?

There are many different reports on which GPCR domain(s) are important for oligomerization. Several types can be recognized: C-terminal tail coiled-coil interaction (e.g. GABAB1 261), N-terminal disulphide bridging (e.g. mGluRs 262,263), and transmembrane (TM) domain interaction. The latter can be separated into either contact interactions (e.g. rhodopsin 344 and D2R 244) or domain swapping (e.g. H1R 199). The interactions are not limited to only one of these types. For both
the GABA<sub>B1</sub> and calcitonin receptor disruption of one specified interaction does not completely abolish GPCR oligomerization. One provocative possibility is that TM domain interactions always play a role in the formation of oligomers.

One of the early reports on GPCR oligomerization came from the group of Maggio. Using chimeric receptors M<sub>3</sub>/α<sub>2</sub>-AR and α<sub>2</sub>-AR/M<sub>3</sub> in which TMI-TMV or TMVI and TMVII are interchanged, they were able to convincingly show GPCR oligomerization via domain swapping. A long IL3 loop, a feature that is lacking in family C GPCRs, in the mechanism of domain swapping was reported crucial. Other evidence in favor of domain swapping came from the histamine H<sub>1</sub> receptor. Co-expression of two defective H<sub>1</sub>R mutants, one with a mutation in TM III and a second with a mutation in TM VI could be reconstituted into a functional receptor via domain swapping. However, similar attempts to rescue defective mutants of the α<sub>1B</sub>-AR and the D<sub>2</sub>R were ineffective, arguing against domain-swapping. Moreover, whereas Maggio and co-workers could not detect dimerization using a M<sub>3</sub> receptor with a truncated IL3 loop, Zeng and co-workers showed oligomerization of a rat M<sub>3</sub> receptor, with a truncated IL3, further undermining the domain swapping theory.

Using evolutionary trace analysis, as well as computer model docking simulations and correlated mutational analysis Gouldson proposes an important role for TM V and VI in the oligomerization interface. Evolutionary trace analysis performed specifically on aminergic GPCRs predicted a potential role for TM IV and V for muscarinic and possibly 5-HT<sub>2</sub> receptors, whereas for other members of the aminergic receptor family TM V and VI were important. This prediction fits the TM I-V TM VI-VII domain swapping strategy. Gouldson predicts a minor role for TM I and TM VII, which would fit the intermediate stage of the domain swapping (Figure 5.2). However, the TM I-V, TM VI, VII domain swapping model, as proposed by Gouldson, is only energetically favorable when bound to an agonist. This is in agreement with the previously reviewed data that oligomers are constitutively assembled in the ER. One may speculate that oligomers can shift between a contact-oligomer interface, when unbound or bound to an antagonist,
and a domain swapping interface when bound to an agonist. However, such a rearrangement would most likely be revealed in BRET assays. As was shown for e.g. the H₄ receptor no change in BRET could be detected upon binding of an inverse agonist, neutral antagonist or agonist. These observations would argue against a situation in which dimers can switch between contact interaction and domain-swapped interaction upon ligand binding.

**Figure 5.2.** Schematic representation of GPCRs forming dimers via domain swapping. The flexible intracellular loop 3 allows for TM VI-VII to be interchanged between the two GPCRs, possibly via an intermediate in which TM I is interacting with TM VII. Figure adapted from 348.

One of the best examples of GPCR oligomerization occurring via contact between the TM domains comes from the rhodopsin receptor. Using atomic force microscopy (AFM) rhodopsin was shown to have contacts between TM IV and V. Rhodopsin dimers form rows as a result of contacts between the cytoplasmic loop connecting TM V and TM VI and TM I and II from the adjacent dimer. Rows assemble into paracrystals through extracellular contacts formed by TM I. However, the dimeric structure seen for rhodopsin may be an artifact of the sample preparation for AFM. Nevertheless, several reports have suggested similar
contact interfaces for aminergic GPCRs (Figure 5.3). The importance of TM I in homo-oligomerization of the $\alpha_{1B}$-AR has been shown convincingly \cite{205, 218, 245}. Carrillo and co-workers employed co-immunoprecipitation and tr-FRET to study the involvement of specific TM domains. They found that TM I and TM IV could self associate, while there was a weaker interaction between TM I/II with TM V-VI \cite{245}. No role was observed for TM VII \cite{245}. In contrast, Stanasila and co-workers reported that TM VII of the $\alpha_{1B}$-AR is involved in the oligomerization interface, albeit to a lesser extent than TM I \cite{218}.

![Schematic representation of an oligomeric complex of aminergic GPCRs. A single row of oligomeric GPCRs can be formed by alternating GPCRs interacting with an adjacent GPCR either via TM IV or TM I. A second row of oligomeric GPCRs is stabilized via an interaction between TM I and TM VI. Figure adapted from 245.](image)

Several reports have shown the importance of TM IV in dopamine D$_2$R homo-oligomerization \cite{244, 298, 299}. Cross linking experiments convincingly showed the involvement of TM IV in the oligomerization interface \cite{244, 298}. However, TM IV is not crucial as oligomers could still be observed when the conformation of TM IV was distorted by insertion of a proline residue \cite{299}. 5-HT$_4$R homodimers are stabilized by disulfide bonds between cysteine residues in TM III and TM IV \cite{353}. Earlier studies showed cysteine residues located in EL2 and EL3 to play an important, albeit not crucial role, in M$_3$ receptor oligomerization \cite{339}. These cysteines are highly conserved among aminergic receptors, compared to the cysteines residues found
in the TM domains of the 5-HT₄R, and can undergo either intra- or inter-molecular cross-linking. Zeng and Wess suggest that in general non-covalent TM domain interactions precedes cross linking. Other TM domains that may play a role in D₃R oligomerization are TM VI and VII. Co-expression of the D₃R with either of these domains was able to dissociate D₃R homo-oligomers. While TM VI appears not to be important in D₁R oligomerization, the involvement of TM VI was shown for the β₂-AR. For the β₂-AR the involvement of TM VI was deduced to the presence of a glycophorin like motif (GXXXGXXXL) within this domain. The glycophorin motif is a potential interface for GPCR oligomerization as has been shown for the yeast α-factor receptor. However, it appears that it is mainly the leucine residue that is crucial for the interaction. Yet, no involvement of the glycophorin motif could be observed for the α₁B-AR, suggesting that this motif is not a general requirement for GPCR oligomerization.

In summary, three TM domains in particular are important within the contact interface model, namely TM I, TM IV and to a lesser extent TM VI. It is noteworthy that these TM domains, especially TM I and TM IV are less involved in ligand binding to aminergic GPCRs, thus allowing them to fulfill different roles such as formation of oligomers. Moreover, amino acids within these TM domains are less conserved than in other TM domains. This lack of conservation may provide a way for receptors to form hetero-oligomers with only a limited amount of other GPCRs, thus creating a certain degree of specificity.

Regarding non TM domain regions, several reports have shown that N-glycosylation is not a requirement for oligomerization. However, inhibition of N-glycosylation resulted in a reduction in β₂-AR oligomerization. In contrast, blocking N-glycosylation increased the formation of the β₁-AR-α₂A-AR hetero-oligomers. The C-terminal-tail is reported to play no role of importance in the formation of α₁B-AR and H₂R oligomers, whereas an electrostatic interaction between an arginine rich region in the IL3 of the D₂R and either a DD region or a phosphorylated serine in the C-terminal tail of the A₂A receptor is involved in the hetero-oligomerization of these two receptors. In general,
based on data from Western blot analysis, two different types of interaction can be appreciated: covalent (dithiothreitol (DTT) sensitive) and strong hydrophobic (SDS insensitive) interactions (Table 5.2). Often Western blot experiments reveal both monomeric and dimeric reactive species. Whether the monomeric species in these blots are SDS sensitive dimers, or native monomers can not be deduced from these experiments.

Table 5.2. Overview of interactions types involved in the formation of oligomers.

<table>
<thead>
<tr>
<th>Interaction type</th>
<th>Receptor oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong hydrophobic (SDS insensitive)</td>
<td>5-HT_{1B}R, 5-HT_{1D}R, D_{1R}, D_{2R}, D_{3R}, H_{1R}, H_{2R}, α_{1B}-AR-MOR, β_{2}-AR-DOR</td>
</tr>
<tr>
<td>Covalent (DTT sensitive)</td>
<td>5-HT_{1B}R, 5-HT_{1D}R, M_{2R}, M_{3R}, D_{1R}</td>
</tr>
</tbody>
</table>

Monomer, dimer or oligomer?

Not only dimers can be identified in Western blots, often immunoreactive species running at approximately four times the weight of a monomeric receptor can be detected. Bands corresponding to these putative tetrameric oligomers have been observed for the D_{1R}, D_{2R}, D_{3R}, M_{2R}, M_{3R}, β_{2}-AR, α_{1B}-AR, 5HT_{1B}, 5HT_{1D}, H_{1R}, H_{2R}. Only for the H_{1R}, H_{2R} and α_{1B}-AR immunoreactive species corresponding to putative trimers have been observed. However, one has to be very careful in interpreting these results. High molecular weight bands can also arise from aggregates between the studied receptor and other proteins, with similar molecular weight. Also the procedure of sample preparation and SDS-page gelelectrophoresis can introduce or break up oligomers. Changes in temperature during sample preparation have been shown to induce oligomerization as well as cause dissociation of oligomers.

Several studies that address the oligomerization state of GPCRs have been performed. BRET saturation curves of β_{2}-AR and muscarinic M_{1R}, M_{2R} and M_{3R} oligomers, fitted a dimer model better than a trimer model as well. However, the M_{2R} is not present exclusively as dimeric or higher oligomeric
structures, but also as monomers \(^{268}\). Three-fluorophore FRET showed that the \(\alpha_{1B}\)-AR is able to form at least trimeric complexes \(^{281}\).

It is difficult to determine whether observed functional effects arise from a GPCR monomer, homo-oligomer or from both. Recent studies show that both rhodopsin monomers as well as oligomers can be activated \(^{356}\). pbFRET and co-immunoprecipitation have been employed to study the G-protein coupling of an inactive 5-HT\(_{2C}\) mutant receptor when co-expressed with the wild-type receptor, revealing that one dimer interacts with only one G-protein, and that two ligands were required to activate this dimer \(^{48}\). This apparent 2:2:1 stoichiometry is in sharp contrast with the traditional belief that GPCRs function in a 1:1:1 stoichiometry. Yet, it is in agreement with the finding that the leukotriene B\(_4\) receptor forms a pentameric structure, consisting of a hetero-trimeric G-protein and a dimeric receptor \(^{47}\). Still, several reports have argued that a monomeric receptors can also be activated by one hetero-trimeric G-proteins \(^{357-359}\). Moreover, in several instances one ligand has been shown to induce internalization of a hetero-dimeric complex, suggesting that one ligand is enough to activate the dimer \(^{289,304,343}\). More studies are required to truly understand the roles and potential differences of receptor monomers and oligomers and the effect of ligand occupation on their activation.

**Effect of ligand binding on oligomerization**

A relatively controversial issue concerning GPCR oligomerization is the effect of ligand binding on the formation of oligomers. The majority of the studies involved in determining the effects of ligand, techniques employ either BRET or FRET. These methods rely heavily on the orientation and conformation of the fluorescent groups. A ligand induced change in conformation, could result in a change in conformation and a concomitant increase or decrease in the distance between the donor and acceptor. Thus, this would give a false notion of an increase or decrease in the amount of oligomers. Similarly, an increase in oligomerization may not be detected
if it would coincide with a conformational rearrangement, increasing the distance between donor and acceptor. This was nicely demonstrated in a study on melatonin receptors. While an agonist induced an increase in BRET signal between MT₁R-Rluc and MT₂R-eYFP, no effect was detected when MT₁R-eYFP were co-expressed with MT₂R-Rluc. This observation clearly demonstrates that a change in BRET signal is not necessarily due to a ligand induced change in the formation of oligomers. In general, for homo-oligomers no ligand effect on oligomer formation or dissociation is observed. Nevertheless, several interesting findings regarding the effect of ligand binding on the formation of hetero-oligomers have been reported. For example, an increase in α₂A-AR-MOR hetero-oligomerization, could be accomplished by activating either receptor, but a decrease was detected when both receptors were activated simultaneously. In contrast, an increase in pbFRET signal was observed for the D₂R-SSTR₅ hetero-oligomer, when the receptors were activated individually as well as simultaneously. A possible explanation for such observations came from the study of hetero-oligomerization between the D₂R and D₁R mutants. Stably formed homo-oligomers of a D₁R mutant, with two mutations in its binding domain (S198A, S199A) in TM V were not affected by binding of the inverse agonist (+)-butaclamol. However, binding of the same ligand to hetero-oligomers between the mutant D₁R and the wild-type D₁R disrupted the hetero-oligomeric complex. In a recent study, Damian and co-workers showed that in a ligand bound leukotriene B₄ receptor homodimer, each individual receptor displayed a different conformation, which remained even when both receptors were occupied by ligands. Apparently conformational changes, caused by ligand binding can have a serious impact on the stability of the oligomer, even if the two receptors within the complex don’t differ much in amino-acid sequence. So even though in most cases ligand binding does not influence GPCR oligomerization, certainly, binding of ligand(s) affects receptor conformation. Hence, it is plausible that in certain cases, e.g. binding of allosteric ligands or binding of two ligands to a hetero-oligomer, may stabilize or destabilize receptor-receptor interactions.
Why are oligomers formed?

Although, so far hetero-oligomerization is only found vital for receptor function for the GABA B 42 and taste receptors 45,46 numerous hetero-oligomers have been reported to have a unique pharmacology 304,308,340. Often receptor trafficking, both transport from the ER to the cell membrane or from the cell membrane to endocytic vesicles by internalization, is altered upon hetero-oligomerization. As previously mentioned, hetero-oligomerization can have a dominant negative 204,285,286 or positive effect 217,287 on receptor cell surface expression. Internalization of GPCRs can change upon hetero-oligomerization. For example, stimulation of β2-ARs has led to co-internalization of MOR 65 and M71 olfactory receptors 287, whereas MOR 65 or M71 287 stimulation results in β2-AR internalization. In contrast, KOR has a dominant negative influence on β2-AR internalization 65. Stimulation of α2A-AR receptors results in internalization of β1-AR receptors 304, whereas activation of α1A-AR leads to α1B-AR internalization 218. Activation of D1R causes internalization of D2R and vice versa 289. D3R also co-internalizes with the adenosine A2aR 321. Additionally, when co-expressed with M3R, carbachol stimulation resulted in increased downregulation of M3R 334.

Besides affecting trafficking of receptors, hetero-oligomers may also exhibit altered signaling properties. For instance, the β2-AR-β2-AR hetero-oligomer can only signal via Gia, while the individual receptors can signal via Gai/o as well 62. Additionally, the recruitment of β-arrestins is reduced for the β2-AR-β2-AR hetero-oligomer, resulting in a decrease of internalization 62. Novi and co-workers showed that the M3R-α2A-AR receptor chimera’s need to oligomerize in order to signal via ERK1/2 phosphorylation 340 and recruit β-arrestins to be internalized 338. Other examples of hetero-oligomers expressing a unique pharmacology are: M2R-M3R 265, β1-AR-β2-AR 308,309, α1B-AR-α1D-AR 302, D2R-D1R 319, D2L-R-SSTR5 272, β1-AR-α2A-AR 304, D3R-A2aR 333 and D1-A1R 295. It would be reasonable to suggest that each hetero-oligomer has a physiological role, either crucial or merely supportive.
Future prospects

The use of chimeric receptors, epitope tagged or fluorescent protein fused receptors has been tremendously valuable for studying GPCR oligomerization in vitro. However, only a limited number of studies have reported on GPCR oligomerization in vivo. Antibodies raised against GPCRs have been an important tool in the detection of oligomers in vivo. Using such antibodies, M2R oligomers have been detected in porcine atrial sarcolemmal membranes 253, M3R 339 and rH3R 204,210 oligomers in rat brain, D3R oligomers in human motor cortical tissue 332, and H4R oligomers in human spleen tissue and PHA blasts 234. While these antibodies were used in Western blotting experiments, labeling these antibodies with europium- or allophycocyanin-conjugates, could potentially allow performing tr-FRET assays in vivo. Also production of antibodies that specifically recognize a hetero-oligomer would be very helpful to study GPCR hetero-oligomerization in vivo. The ability to study GPCR oligomerization in vivo using different and more advanced techniques would undoubtedly aid in understanding their physiological relevance, and potentially linking them to pathological conditions. For example, in pre-eclamptic hypertensive women an increase in hetero-dimers between the angiotensin AT1 and bradykinin B2 receptors has been shown to result in a concomitant increase in responsiveness to angiotensin II 361. Designing ligands that target hetero-oligomers may be successful as therapeutics in these cases. Recently, a bivalent ligand that is selective for the DOR-KOR heterodimer has been developed 197. Although it is very difficult to produce these (specific) bivalent ligands, as can be seen from the attempts to produce bivalent ligands for the 5-HT4 receptor 362, these ligands provide compelling tools to study GPCR oligomerization in vivo. Besides bivalent ligands, it may be possible to develop small, monovalent hetero-oligomer selective ligands. Ligands that can apparently recognize different populations of receptors (monomer vs oligomer) have already been discovered. For e.g. the D2Rs [125I]azidophenethylspiperone was shown to detect only monomeric receptors while [125I]-4-azido-5-iodononapride can also detect dimeric receptors 325. Similarly [3H]quinuclidinylbenzilate labeled a different population of M2Rs than N-[3H]methylscopolamine or [3H]AF-DX 384. For the H1Rs [3H]mepyramine has been shown to label a different population of receptors...
compared to $[^3]H/]H2-PAT$, that possibly recognized only dimeric receptors 199. Recently SKF83959 was identified as a specific agonist for the D1R-D2R hetero-oligomer 319. Perhaps some orphan ligands, like e.g. CXCL14 (BRAK), do not recognize a single GPCR but only a hetero-dimer, explaining why these ligands have not been de-orphanized yet. Although increasingly more information on GPCR oligomerization is becoming available, still many aspects are not fully understood (Figure 5.4).

**Figure 5.4.** Overview of GPCR oligomerization ontogeny and trafficking. A, GPCR oligomers already form in the endoplasmatic reticulum. B, dominant negative GPCRs may block surface trafficking, while other GPCRs may facilitate surface expression by hetero-oligomerization. C, GPCR oligomers are constitutively present at the cell surface. D, GPCRs can be co-internalized after agonist stimulation, or internalization can be inhibited through hetero-oligomerization. Questions remaining: ?, What are the specific roles of the monomers and oligomers. ??, can GPCRs within different oligomeric complexes exchange partners while on the cell surface. ???, What determines through which G-protein ($G_{i\alpha}$, $G_{s\beta\gamma}$, $G_{q\alpha}$ or $G_{12\alpha}$) will be signaled if an activated hetero-oligomer binds only one G-protein.

Extensive study on rhodopsin has shown that the receptor can be activated as a monomer 356,358 as well as an oligomer 356. Recent papers have also shown that one G-protein can be activated by one receptor dimer 47,48. However, only few studies have tried to determine the percentage of receptors present as dimers. Western
blot and BRET analysis estimated that 45-85% of receptors are interacting as dimers\textsuperscript{301,334,339}. The question arises what the physiological purpose is of having populations of monomers and oligomers. Work of Jastrzebska and co-workers suggest that oligomers are activated at a higher rate then monomers\textsuperscript{366}. Are receptor monomers primarily created by break up of oligomers, waiting to form new oligomeric complexes, or are they transported to the cell surface as monomers, having to fulfill different tasks than receptor oligomers (Figure 5.4)? In order to more accurately predict pharmacology of dimers it is necessary to understand the dynamics of dimerization. Regarding these dynamics of GPCR hetero-oligomerization, it would be interesting to determine whether oligomers, can dissociate while on the cell surface (Figure 5.4). If so, this would allow for the exchange of partners within hetero-oligomers complexes. Computer simulation of GPCR arrangement predicted that in such a situation homo-dimers or hetero-dimers would form clusters\textsuperscript{363}. However, if oligomers are formed solely intracellular and do not exchange partner during their lifespan, this would raise new questions. For example, in order for the $\beta_2$-AR to exert the dominant-positive effect on cell surface translocation of $\alpha_1D$-AR\textsuperscript{217} and M71\textsuperscript{287} both receptors would have to be transcribed and translated at the same time. It would therefore be interesting to investigate if for example expression of $\beta_2$-ARs coincides with the expression of M71 olfactory receptors or $\alpha_1D$-ARs to determine if this effect is indeed physiologically relevant. Recently, Law and co-workers used an inducible promoter to express $\delta$-opioid receptors in cells endogenously expressing $\mu$-opioid receptors\textsuperscript{364}. The use of inducible promoters could be used to specifically address the question of oligomer formation at the cell surface. By placing two receptors under control of different inducers, the receptors could be expressed consecutively instead of simultaneously, excluding that any observed oligomers would have been formed during formation and transport. Other techniques, such as eBRET\textsuperscript{278} may also be used to investigate whether homo-dimers or hetero-dimers break up at the cell surface to exchange partners, by using the EnduRen\textsuperscript{TM} substrate on cells expressing receptors fused to Renilla luciferase and eGFP, and studying the BRET signal over a prolonged amount of time. Finally, if indeed one dimer binds only one G-protein, this raises exciting questions from a molecular pharmacological point of
view. For example, it would be interesting to determine to which class of G-proteins \( (G_{\alpha i}, G_{\alpha s}, G_{\alpha q} \text{ or } G_{\alpha 12}) \) a hetero-dimer, made up of receptors that individually couple to different G-proteins, would couple upon activation and what the driving force is behind this coupling (Figure 5.4).

In conclusion, over the last twenty years the study of GPCR oligomerization has increased steadily. This has resulted in a definite increase in the understanding of the mechanism behind the formation and the cellular role of these oligomers. However, still many questions remain unanswered. In order to take the research of GPCR oligomerization to the next level, more \textit{in vivo} studies are required. The development of new techniques and new tools will be of crucial importance to obtain new insights into GPCR oligomerization and elucidate the physiological importance and dynamics of this phenomenon.
CHAPTER 6

Hetero-oligomerization of the human histamine H₄ receptor and the human cytomegalovirus encoded chemokine receptor US28

Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
Centre for Integrative Neuroscience (CINS), School of Biological & Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, England
ABSTRACT

The histamine H₄ receptor (H₄R); a member of the G protein-coupled receptor (GPCR) family and is predominantly involved in inflammatory responses, such as chemotaxis of leukocytes and the release of chemotactic peptides. Human cytomegalovirus (HCMV) is a β-herpesvirus, which is believed to contribute to the pathology of chronic inflammation and several other diseases, and is able to infect leukocytes. The four GPCRs encoded by the HCMV genome are homologous to chemokine receptors. The most well studied of these is US28, which has been previously reported is able to interact with the human chemokine receptor CCR1 (J. Biol. Chem. 279:5152-5161). As US28 and the H₄R are co-expressed on leukocytes, and we have previously shown that the H₄R can homo-oligomerize (Mol. Pharmacol 70:604-615), we set out to investigate if US28 can also interact with the H₄R. We found that in a heterologous system, in which H₄R is co-expressed with US28, additional signaling pathways for the H₄Rs are unmasked. Using biochemical and biophysical assays, we were able to show that H₄R can hetero-oligomerize with US28. Therefore the change in signal transduction may be due to hetero-oligomerization between H₄R and US28. These data suggest that US28 may exert part of its physiological influences by hetero-oligomerization with the H₄R.

INTRODUCTION

It is now well established that GPCRs can form homo- and hetero-oligomers, and we have recently shown homo-oligomerization of the human H₄R in lymphocytes. The H₄R is an aminergic GPCR that is expressed almost exclusively in cells belonging to the hematopoietic lineage. Based on the expression pattern and the ability of the H₄R to induce chemotaxis of eosinophils and mast cells, this receptor has been linked to inflammatory diseases, such as allergy and rheumatoid arthritis. Recently, evidence for a role of H₄Rs in cancer has emerged as well.
Chemokine receptors are renowned for their role in inflammation. Several of these peptidergic receptors, e.g. CXCR1, CXCR2, CXCR4, and CCR5, have been shown to form homo-oligomers as well as hetero-oligomers. Several viruses encode chemokine receptors, including Epstein-Barr virus, Kaposi’s sarcoma associate herpesvirus and human cytomegalovirus (HCMV). Infection with HCMV has been linked to colon cancer and breast cancer. The best characterized chemokine receptor encoded by the HCMV is US28. US28 shares highest homology with CCR1 and CX3CR1 and is capable of binding a broad spectrum of chemokines such as CCL2, CCL3, CCL4, CCL5 and CX3CL1. Recently, we reported that the Gαq/11-coupled US28 receptor potentiates signaling via the Gαi/o-coupled CCR1 receptor, which might be a common feature among GPCRs.

The H4R is not only expressed in the same tissue and cells as chemokine receptors and acting as chemotactic receptor, but the H4R also plays a role in cytokine and chemokine release. Moreover, Nakayama and co-workers reported that CCL16 (LEC) is able to bind and signal via the H4R. Hetero-oligomerization between peptidergic and aminergic receptors has already been reported, as both the δ- and κ-opioid receptor could form hetero-oligomers with the β2-adrenergic receptor. We previously reported on the synergistic effects between US28 and CCR1, which might possibly occur through heterodimerization. As both US28 and H4Rs are preferentially expressed on leukocytes, we set out to investigate the effects of co-expression of the H4R and US28.

Herein we report on the homo-oligomerization of US28 as well as on the hetero-oligomerization of US28 with hH4Rs as determined using both biochemical (co-immunoprecipitation) and biophysical approaches (BRET, tr-FRET). Co-expression of H4Rs with US28 affects the pharmacology of the H4R, causing a reduction in H4R Bmax-values. In addition, co-expression of the H4R with US28 reveals H4R mediated NF-κB and InsP3 signaling.
RESULTS

The viral chemokine US28 receptor forms homo-oligomers and hetero-oligomers with the H4R.

We have previously shown that the H4R can form homo-oligomers and that H4R splice variants act as dominant negative by forming hetero-oligomers with the full length H4R. We have also shown previously that constitutively active Gq/11-coupled receptors constitute a regulatory switch for signal transduction of Gi/o-coupled receptors. As both the Gq/11-coupled H4R and the Gq/11-coupled US28 play a role in inflammatory responses we were interested to see whether the H4R and US28 could form hetero-oligomers and/or interact with each other on the level of receptor signaling. To investigate whether hetero-oligomerization occurs between the H4R and US28 bioluminescent resonance energy transfer (BRET) measurements were performed. To this end, C-terminal tagged eYFP (US28-eYFP) or Renilla luciferase (US28-Rluc) US28 were constructed. The US28-Rluc and US28-eYFP were paired and co-expressed with either H4R-eYFP or H4R-Rluc, respectively. Upon addition of coelenterazine, the substrate for Renilla luciferase, BRET measurements were performed. A clear BRET signal was observed when US28-Rluc was co-expressed with H4R-eYFP or when US28-eYFP was co-expressed with H4R-Rluc (Figure 6.1). Additionally, a BRET signal was observed in cells co-expressing US28-Rluc and US28-eYFP (Figure 6.1). Both the BRET signals for the US28 homo-oligomer and US28-H4R hetero-oligomer are stronger than for the H4R homo-oligomer.

To further confirm the existence of H4R-US28 hetero-oligomers and to determine whether the hetero-oligomer is also present on the cell surface tr-FRET measurements were performed. For this purpose COS-7 cells co-expressing N-terminally FLAG-tagged histamine H4R (FLAG-H4R) and N-terminally HA-tagged US28 (HA-US28) were incubated with Europium (Eu3+) labelled anti-FLAG antibodies or a combination of the Eu3+ labelled anti-FLAG and allophycocyanin (APC) labelled anti-HA antibodies. As a control, cells solely expressing FLAG-H4R or HA-US28 were mixed and exposed to the two antibodies. A significant tr-FRET signal was observed in COS-7 cells co-expressing both FLAG-H4R and HA-US28.
compared to the signal obtained from cells only expressing either receptor that were mixed prior to incubation with the antibodies (Figure 6.2). To determine the specificity of the hetero-oligomerization between the H₄R and the US28 receptor, FLAG-H₄R were co-transfected with a N-terminally 3xHA-tagged human chemokine receptor CCR2 (3xHA-CCR2). However, no significant tr-FRET signal could be obtained for this combination.

**Figure 6.1.** Evaluation of hetero-oligomerization between the H₄R-Rluc US28-eYFP receptors by BRET using the co-expression of Renilla luciferase (Rluc) and eYFP C-terminal receptor-fusion proteins. BRET ratios for transiently transfected COS-7 cells co-expressing the H₄R-Rluc and H₄R-eYFP, US28-Rluc and US28-eYFP receptors or H₄R-Rluc and US28-eYFP. Cells expressing the indicated receptor-fusion proteins were exposed to 5 μM coelenterazine after which resonance energy transfer was measured. Ratios are expressed as mean ± S.E.M. from at least three experiments each performed in triplicate.
Figure 6.2. Evaluation of hetero-oligomerization between the FLAG-tagged H₄R and HA-tagged US28 receptors by tr-FRET. Transiently transfected COS-7 cells co-expressing the H₄R and US28 receptors were incubated for 2 hours with the Eu³⁺-labelled anti-FLAG and APC-labelled anti-HA antibodies. A, tr-FRET in COS-7 cells co-expressing FLAG-US28 and HA-US28 compared to COS-7 cells co-expressing FLAG-H₄R and HA-H₄R. B, tr-FRET in COS-7 cells co-expressing FLAG-H₄R and HA-US28 or 3xHA-tagged CCR2. Data are normalized for the tr-FRET signal obtained from a mixture of COS-7 cells that individually expressing the indicated constructs. Data shown are average of at least three experiments each performed in triplicate.

Both BRET and tr-FRET assays are based on resonance energy transfer. Consequently, observed signals may arise from receptors that are in close proximity of each other, but do not physically interact to form oligomers. To establish whether the H₄R physically interacts with US28 co-immunoprecipitation assays were performed. Anti-FLAG coupled sepharose beads were used to precipitate FLAG-H₄R from HEK 293 cells co-expressing HA-US28 and FLAG-H₄R. Subsequent Western blotting experiments performed with anti-HA antibodies revealed the presence of US28 in the precipitate (Figure 6.3, lane 3). No HA-US28 was immunoprecipitated with control sepharose beads that were not coupled to antibodies (Figure 6.3, lane 2).
Chapter 6

Figure 6.3. Immunoprecipitation evidence for H₄R receptor interaction with US28. HEK 293 cells were co-transfected with equal amounts of cDNAs encoding FLAG-H₄R and HA-US28, harvested 40h post transfection, solubilised with 1% (v/v) Triton-X100 and subjected to immunoprecipitation with anti-FLAG sepharose or sepharose control beads for 2h at 4°C. Following washing, bound material was eluted using SDS-PAGE sample buffer and analysed by immunoblotting. Immunoblots were probed with anti-HA antibody. A single diffuse species was identified consistent with the US28. Lane 1 solubilized cells, Lane 2 cells immunoprecipitated with sepharose beads, Lane 3 cells immunoprecipitated with anti-FLAG sepharose beads.

Co-expression of US28 decreases number of H₄R binding sites.

To investigate the effect of hetero-oligomerization between US28 and the H₄R on their pharmacology, [³H]histamine radioligand binding was performed on membranes of COS-7 cells transfected with H₄R and US28 cDNA in a 1:1 ratio. [³H]histamine bound to the COS-7 cells co-expressing the H₄R and US28 according to a one site, saturable model with a dissociation constant (Kᵣ) that is not significantly different than the value obtained when only the H₄R is expressed in COS-7 cells (40 ± 13.1 vs 17 ± 2.1 nM). However, the Bₘₐₓ, the maximal amount of H₄R labeled by [³H]histamine (0.84 ± 0.2 pmol/mg protein) decreased by 35% (65.1% ± 17.7, n=3) upon co-expression with US28 compared to COS-7 cells expressing only the H₄R. To further investigate the effect of US28 co-expression on H₄R pharmacology radioligand displacement assays were performed. [³H]histamine radioligand binding was performed on membranes of COS-7 cells expressing the HA-H₄R in the presence or absence of US28. The selective H₄R agonist 4-
methylhistamine, partial agonist clobenpropit, neutral antagonist JNJ 7777120 and inverse agonist thioperamide were used to displace [3H]histamine. On COS-7 cells expressing US28, no [3H]histamine binding was observed (data not shown). The affinity of the H₄R ligands for the H₄R does not significantly (p>0.05) change when US28 receptors are co-expressed (Table 6.1, Figure 6.4B).

Table 6.1. Characterization of the H₄R expressed individually or co-expressed with US28 in transiently transfected COS-7 cells. The Kᵦ values for [3H]histamine, H₄R expression (Bmax) and the pKᵦ values of 4-methylhistamine (4-Me-HA), clobenpropit, JNJ7777120 and thioperamide were determined by [3H]histamine saturation and displacement binding assays. COS-7 cells were transfected in a 1:1 ratio with cDNA encoding the H₄R and US28. The values are expressed as mean ± SEM of at least three independent experiments.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kᵦ (nM)</th>
<th>Bmax (%)</th>
<th>[3H]histamine</th>
<th>pKᵦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄R</td>
<td>17.1 ± 2</td>
<td>100 ± 3.3</td>
<td>7.1 ± 0.1</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>H₄R + US28</td>
<td>40.1 ± 2</td>
<td>59.1 ± 4.3</td>
<td>6.9 ± 0.1</td>
<td>7.5 ± 0.2</td>
</tr>
</tbody>
</table>

Finally, ligand induced dissociation of [3H]histamine from COS-7 cells individually or co-expressing H₄R and US28 was investigated. In COS-7 cells expressing H₄R the selective H₄R JNJ 7777120, but not CCL5 was able to dissociate [3H]histamine (Figure 6.4C). With the exception of a reduction in Bmax similar results were obtained in COS-7 cells co-expressing H₄R and US28 (Figure 6.4C). No difference was observed in dissociation constants (7.0 ± 2.0 vs 5.6 ± 1.6 min).
Figure 6.4. Dominant negative effect of US28 receptors on \[^{3}H\]histamine binding to H\textsubscript{4}R. A, Saturation of binding to H\textsubscript{4}R receptors expressed individually (●) or co-expressed with the US28 (○) in COS-7 cells. B, Displacement of \[^{3}H\]histamine by the selective H\textsubscript{4}R agonist 4-methylhistamine in COS-7 cells. C, Dissociation of \[^{3}H\]histamine from COS-7 cells individually expressing H\textsubscript{4}R (closed symbols) or co-expressing with the US28 (open symbols) induced by the H\textsubscript{4}R antagonist JNJ 7777120 (circle) or US28 selective ligand CCL5 (square) Representative experiments are shown.
Surface labeling shows large intracellular population of
H₄R-US28 hetero-oligomers.

The findings of an apparent reduction in H₄R expression when co-expressed with US28 together with the ability of these receptors to form hetero-oligomers prompted us to further study the localization of the H₄R and US28 when co-expressed. US28 has been reported to be rapidly constitutively internalize. Surface labeling of HEK 293 cells expressing US28 showed the majority of US28 receptors to be located intracellular, with only a small percentage present on the cell membrane (Figure 6.5A).

**Figure 6.5.** Surface Biotinylation of US28 and H₄R. HEK 293 cells were transfected with either cDNAs encoding H₄R or HA-US28 alone, or co-transfected with equal amounts of H₄R and HA-US28. Intact cells were biotinylated for 15 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin, washed and homogenised. Biotinylated surface fractions were isolated by streptavidin chromatography, and analysed by immunoblotting. A, HA-US28 transfected alone and immunoblot probed with anti-HA antibody. A single diffuse species labelled consistent with the size of HA-US28. B, H₄R alone or co-transfected with HA-US28. Immunoblot probed with rabbit anti-hH₄R antibody (Two major diffuse protein species labelled corresponding to dimeric and monomeric glycoproteins) or mouse anti-beta-actin antibody. The lack of beta-actin signal in re-probed surface fractions demonstrates successful fractionation. I = Intracellular fraction; S = Surface fraction.
The H₄R expressed in HEK 293 cells is also present at the cell surface (Figure 6.5B, lane 2). However co-expression with US28 results in a decrease in surface localized H₄R to undetectable proportions (Figure 6.5B, lane 4). β-actin controls show that this result is not caused by an overall decrease in protein expression (Figure 6.5C). Additionally, the β-actin control show that the fractionation was successful as β-actin is not expressed on the surface.

**Co-expression of H₄R and US28 unmasks H₄R mediated NF-κB signaling.**

The functional analysis of co-expression of the H₄R and US28 receptors was performed using a NF-κB-luciferase reporter gene assay in transiently transfected HEK 293T cells. In line with earlier findings, US28 exhibits constitutive NF-κB signaling when expressed in HEK 293T cells (5-fold versus mock) (Figure 6.6). The H₄R does not signal via NF-κB, as no constitutive activity or ligand induced changes in NF-κB signaling are observed versus mock HEK 293T cells (Figure 6.6). On HEK 293T cells expressing US28 receptors no effect on NF-κB signaling is observed after stimulation with 10 μM of the selective H₄R agonists 4-methylhistamine and clobenpropit, the selective H₄R antagonist JNJ 7777120 and the H₄R inverse agonist thioperamide (Figure 6.6). However, in HEK 293T cells co-expressing both US28 and H₄R an increase on top of the constitutive US28 signaling is observed compared to HEK 293T cells expressing US28 receptor alone (100 vs 153%). Stimulation with the H₄R agonists 4-methylhistamine and clobenpropit resulted in a clear induction of NF-κB signaling (Figure 6.6). Surprisingly, the H₄R antagonist JNJ777120 increased NF-κB signaling (p<0.005), behaving like a partial agonist. The H₄R inverse agonist thioperamide reduced the H₄R mediated NF-κB signaling back to the level observed in HEK 293T cells expressing US28 alone (Figure 6.6). Co-stimulation of HEK 293T cells co-expressing US28 and H₄R with 4-methylhistamine and JNJ 7777120 resulted in signaling comparable to stimulation with JNJ 7777120 alone (Figure 6.6).
Figure 6.6. Co-expression with US28 reveals H₄R mediated NF-κB signaling in HEK 293T cells. HEK 293T cells expressing either H₄R, US28 or co-expressing H₄R and US28 receptors were stimulated with 10 μM of the selective H₄R agonists 4-methylhistamine and clobenpropit, the selective H₄R antagonist JNJ 7777120, the H₄R inverse agonist thioperamide or a combination of 4-methylhistamine and JNJ 7777120. NF-κB signaling was measured in a NF-κB-luciferase reporter assay. Data is normalized against signal from non-stimulated HEK 293T cells expressing only US28 receptors.

Activation of the H₄R induces InsP₃ accumulation when co-expressed with US28.

Since analysis of NF-kB signaling involves a downstream mechanism, we set out to investigate the effect of H₄R and US28 co-expression in an upstream signaling event such as inositol phosphate production. To study the consequence on functional effect of co-expression of the H₄R and US28 receptors an [³H]inositol phosphate (InsP₃) assay was employed using transiently transfected COS-7 cells. US28 receptors exhibit constitutive InsP₃ accumulation when expressed in COS-7 cells (3-fold vs mock). Although the H₄R is not Gα₁₁-coupled, the H₄R is known to induce Ca²⁺-release via βγ-subunit activation of PLC and may therefore cause InsP₃ accumulation. No H₄R ligand induced InsP₃ accumulation was observed in COS-7 cells expressing H₄R (Figure 6.7). The tested H₄R ligands are selective and do not produce InsP₃ accumulation in COS-7 cells expressing US28 receptors alone (Figure 6.7). In cells co-expressing H₄R and US28 receptors, stimulation
with 10 μM of the H₄R agonist 4-methylhistamine results in a small (105 ± 17 vs 120 ± 17 %) but significant (p<0.01) increase in InsP₃ accumulation (Figure 6.7). This 4-methylhistamine mediated InsP₃ production can be effectively inhibited by the H₄R inverse agonist thioperamide (Figure 6.7).

**Figure 6.7.** Activation of H₄R induces InsP₃ accumulation in COS-7 cells when co-expressed with US28. COS-7 cells expressing either H₄R, US28 or co-expressing H₄R and US28 receptors were stimulated with 10 μM of the selective H₄R agonists 4-methylhistamine, the H₄R inverse agonist thioperamide or co-stimulated of 4-methylhistamine with thioperamide. InsP₃ accumulation was measured in a [³H]InsP₃ accumulation assay. Data is normalized against signal from non-stimulated COS-7 cells expressing only US28 receptors.

**DISCUSSION**

HCMV is a species-specific virus belonging to the beta herpesvirus subfamily. HCMV is common in all human populations, with a seroprevalence ranging from 50 to 80% ³⁸⁵. Leukocytes are a major site of HCMV latency ³⁸⁶,³⁸⁷. CMV infection has a potential role in atherosclerosis ³⁸⁸ and recently we have shown that the CMV-encoded chemokine receptor US28 might play a role in tumorigenesis ³⁸⁹. US28 promiscuously binds several chemokine ligands, including CCL2, CCL3, CCL4, CCL5 and CX3CL1 ³⁷⁹,³⁸⁰, and US28 is rapidly internalized ³⁸³, which has led to the suggestion that US28 acts as a chemokine scavenger ³⁹⁰. This chemokine sequestration may be a strategy of US28 to evade immune surveillance. In this
study we describe another strategy in which US28 takes control of the human immune machinery to increase its signaling output and potentially influence immune responses, such as by modulating chemotactic responses of leukocytes i.e. by removing chemotactic receptors from the cell surface, US28 may prevent the HCMV infected cell to migrate to a site of inflammation.

The latent presence of CMV and US28 mRNA in infected leukocytes brought us to investigate the interaction between US28 and H4Rs. The H4R is expressed almost exclusively in hematopoietic cells and H4R activation can induce chemotaxis of eosinophils and mast cells. The H4R is involved in mediating the release of several interleukins and chemokines. Moreover, activation of the H4R may also cause modulation of cytokine-induced JAK/STAT signaling. This clearly demonstrates that the H4R can interact and exert influence on the chemokine system.

One way for GPCRs to interact with each other is by means of hetero-oligomerization. Several chemokine receptors have been shown to form homo-oligomers as well as hetero-oligomers. We have recently shown that the H4R can form homo-dimers. The possible existence of US28 homo-dimers has also been predicted by the presence of high molecular weight species in Western blot assays. By means of BRET and trFRET we determined that US28 forms homo-oligomers that are present at the cell surface of living cells. Interestingly, we could detect strong BRET and trFRET signals between the H4R and US28, suggesting the existence of H4R-US28 hetero-oligomers. This was further supported by the fact that HA-US28 could be immuno-precipitated by FLAG-H4R. This data suggest the presence of three populations when H4R and US28 are co-expressed; a population of H4R monomers/homo-oligomers, a population of US28 monomers/homo-oligomers and a population of H4R-US28 hetero-oligomers.

When we recombinantly co-expressed H4Rs with US28 we detected a decrease in binding sites for [3H]histamine, an H4R radioligand. However, the remaining H4R binding sites retained their H4R pharmacology as determined by radioligand displacement and dissociation assays. Recently, we demonstrated that Ga(i/o)-coupled signaling may be unmasked when co-expressed with the constitutively active Ga(q/11) coupled receptors. Co-expression of Ga(q/11) coupled US28 and
$G_{\alpha_{i/o}}$-coupled CCR1 results in an robust increase in the $G_{\alpha_{i/o}}$-mediated response to CCL5 in a NF-κB reporter-gene assay \(^{381}\). Since the H₄R is also coupled to $G_{\alpha_{i/o}}$ proteins, we were interested in the possible effects of co-expression of H₄R and US28 in a NF-κB reporter gene assay, as well as in an $[^{3}H]$inositol phosphates accumulation assay. We found that co-expression of H₄R results in an increase in basal US28-mediated NF-κB signaling. This increase is most likely due to the constitutive activity of the H₄R \(^{115}\), since it could be completely blocked with the H₄R inverse agonist thioperamide. Additionally, when co-expressed with US28 stimulation with selective H₄R agonists, such as 4-methylhistamine and clobenpropit resulted in a clear increase in NF-κB signaling. Interestingly, the H₄R partial agonist clobenpropit \(^{115}\) induced a stronger increase then the H₄R full agonist 4-methylhistamine. Moreover the antagonist JNJ 7777120 \(^{115}\) behaved as a weak partial agonist in this co-expressed system, suggesting that these H₄R ligands have a different pharmacology when co-expressed with US28. While less robust than in the NF-κB-reporter gene assay, activation of H₄R with 4-methylhistamine resulted in InsP₃ formation only when co-expressed with US28, but not when expressed alone.

We put forward two possible mechanisms for the observed effects. 1) The H₄R forms hetero-oligomers with US28 that have an altered pharmacology in signaling. Moreover the H₄R is rapidly co-internalized by US28, based on the low surface expression of H₄R when co-expressed with the rapid and constitutive internalizer US28 \(^{383}\). 2) Hetero-oligomerization between the H₄R and US28 retains the both receptors intracellular, however through downstream cross-activation, (constitutively) activated H₄R influences US28 signal transduction (figure 6.8).

More experiments are necessary to determine whether one of the mechanisms or a combination of both can be fully explain the observed effects. Under both circumstances we hypothesize that US28 may hijack the H₄R for its own advantage. By reducing the H₄R binding sites, through hetero-oligomerization and ER retention or subsequent rapid internalization, H₄R-mediated signaling, e.g. chemotaxis, may be reduced. Thereby US28 may evade the immune response of the cells upon encountering chemotactic ligands. While the chemotactic properties of histamine are debatable \(^{165}\), through activation of H₄R, histamine has been
shown to prime leukocytes to potentiate the chemotactic effect of several chemokines e.g. CCL7/MCP-3, CCL11/eotaxin and CCL24/eotaxin-2\textsuperscript{165,167}.

Secondly, in cells in which the H\textsubscript{4}R is co-expressed with the US28, US28 may increase its physiological output. Further studies should be performed to investigate to what extent US28 is able to interfere with H\textsubscript{4}R signal transduction e.g. by determining EC\textsubscript{50} values for histaminergic and chemokine ligands in signaling assays and by determining receptor trafficking in more detail via fluorescence techniques or e.g. biotin protection assays\textsuperscript{393}. Also cells endogenously expressing H\textsubscript{4}R which have been infected with HCMV might be employed to study the interaction between H\textsubscript{4}R and US28 under more physiologically relevant conditions. Additionally, it will be very interesting to study the effects of US28 co-expression on H\textsubscript{4}R mediated chemotaxis.
MATERIALS AND METHODS

Materials. Reagents for tr-FRET were from Cis Bio international (Bagnols-sur-Cèze Cedex, France). Chloroquine diphosphate, DEAE-dextran (chloride form) and histamine (2-[4-imidazolyl]ethylamine hydrochloride), was purchased from Sigma (USA). Calf serum was purchased from Integro BV (Dieren, The Netherlands). Polyethylenimine (linear, MW~25.000) was from Polysciences, Inc (Warrington, PA, USA). D-luciferin was from Duchefa (Haarlem, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Life technologies (Merelbeke, Belgium). Cell culture plastics were from Greiner Bio-one (Wemmel, Belgium). Oligonucleotides were purchased from Isogen Bioscience (Maarsen, The Netherlands). PfuTurbo® DNA polymerase was purchased from Stratagene (La Jolla, USA). Restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). Thioperamide, clobenpropit, 4-methylhistamine and JNJ 7777120, were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam. Gifts of expression vector pcDEF3 (Dr. J Langer), and are greatly acknowledged. Wild type human H4R in pcDNA3.1 was purchased from Guthrie cDNA resource center (Sayre, PA). The vector was subcloned into the pcDEF3 using BamHI/XbaI sites.

Cell culture and transfection. COS-7 African green monkey kidney and HEK 293T human embryonic kidney cells were maintained at 37°C humidified in 5% CO2/95% air atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 50 IU/ml of penicillin and 50 μg/ml streptomycin and grown in 100 mm dishes. COS-7 Cells were transiently transfected using the DEAE-dextran method as previously described 85. HEK 293T cells were transfected using the PEI transfection method as described previously 382. When necessary the total amount of DNA transfected was maintained constant by addition of pcDEF3.

BRET assay. Forty-eight hours post-transfection cells were detached with trypsin and washed twice with PBS. Approximately 50,000 cells per well were distributed in white bottom 96-wells microplates. Coelenterazine was added to a final concentration of 5 μM and readings were collected immediately following this addition using a Victor® 1420 Multilabel Counter allowing signal detection at 460 nm and 530 nm.

Time resolved -FRET assay. tr-FRET assays were performed using a combination of both Eu3+-labeled and allophycocyanin-labeled anti-FLAG and anti-HA antibodies as described previously 234. Briefly, tr-FRET was assessed in 1 x 10^6 whole COS-7 cells transiently expressing the appropriate HA and FLAG tagged receptors. Cells were incubated in PBS containing 50% FCS (v/v), 0.8 nM of Eu3+-labelled antibody, and 8 nM of allophycocyanin-labelled antibody for two hours at room temperature on a rotating wheel, after which the membranes were washed twice with PBS. The final pellet was resuspended in 50 μl PBS and transferred to a 384-microtiter plate. Energy transfer was measured by exciting the Eu3+ at 320 nm and monitoring the allophycocyanin emission for 500 μs at 665 nm using a Novostar (BMG Labtechnologies) configured for tr-fluorescence after a 100 μs delay.
Immunoprecipitation assay. HEK 293 cells were transfected with cDNAs encoding FLAG-H4R(390) with or without HA-US28 using a lipofectamine method. Cell samples were solubilised with 1% Triton-X100 in 50 mM Tris-HCL pH 7.4 and incubated with 50 μl 50% (w/v) slurry of anti-FLAG Sepharose beads (Sigma, UK) for 2 hrs at 4°C. Following incubation, beads were centrifuged at 9000rpm at 4°C for 5 min. The supernatant was retained (unbound fraction) and the beads washed in 50 mM Tris-HCL pH7.4/Triton X100 and bound fraction isolated by elution in SDS PAGE Sample buffer/50mM DTT at 50°C for 30 min. Supernatants following centrifugation at 9000 rpm were retained (bound fraction) for analysis.

Immunoblotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 6 % or 7.5 % polyacrylamide slab gels under reducing conditions. Samples of HEK 293 cells (20-50 μg protein) were prepared using a chloroform/methanol method of protein precipitation, and immunoblotting was performed as previously described. Immuno blots were probed with anti-H4R 374-390 antibody at a concentration of 0.5 μg/ml. Blots containing FLAG- or HA-tagged receptors were probed with primary antibodies, mouse anti-FLAG (1.5 μg/ml) or rat anti-HA (1 μg/ml), respectively. Horseradish peroxidase conjugated goat anti-mouse or anti-rat antibodies (1:2000-5000) were used as secondary antibodies (Little Chalfont, Buckinghamshire, England).

Surface Biotinylation assay. HEK 293 cells were transfected with individual cDNAs encoding H4R(390) or US28, or co-transfected with H4R(390) with equal amounts of US28 using lipofectamine method. The medium was carefully removed 24-40 hours post transfection and cells were washed with ice-cold PBS/4 % (w/v) sucrose (3x1 ml per 35mm dish). The cells were then incubated for 15 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin in ice-cold PBS/4 % (w/v) sucrose (0.5 ml/dish) with gentle shaking. Subsequently, cells were washed once with ice-cold PBS/4 % (w/v) sucrose (1ml/dish), and incubated for 10 min at 4°C with quenching buffer (192mM glycine, 4% (w/v) sucrose in 50mM Tris-HCl pH 8.0) (0.5 ml/dish). Cells were scraped into PBS/4 % (w/v) sucrose and spun at 6000 rpm for 2 min. This wash procedure was repeated on a further occasion and the cells were homogenised in 1% (w/v) SDS in lysis buffer (50mM Tris-HCl, pH 8.0/2 mM EDTA/1% Triton-X100 The samples were then diluted five-fold in lysis buffer (without SDS), and incubated with 20 μl 50% (w/v) slurry of streptavidin beads for 2 hrs at 4°C. Following incubation, beads were centrifuged at 9000rpm at 4°C for 5 min. The supernatant (intracellular fraction) was retained and the beads washed in lysis buffer. The bound fraction was isolated by elution in SDS PAGE Sample buffer/50mM DTT at 50°C for 30 min. Supernatants following centrifugation at 9000 rpm were retained (surface fraction) for analysis.

Radioligand binding studies. Cells used for radioligand binding studies were harvested 48h post transfection and homogenized in ice cold hNaR binding buffer (50 mM Tris, pH 7.4). For saturation isotherms cell membrane homogenates were incubated at 37°C for 60 minutes with 0-150 nM [3H]histamine in a total assay volume of 200 μl. For competition binding assays the cell homogenates were incubated at 37°C for 60 minutes with 0.1-10.000 nM ligand in the presence of ~15 nM [3H]histamine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice cold
hH₄R binding buffer. Bound radioactivity was separated by filtration through GF/C filterplates (Perkin Elmer) that had been treated with 0.3% PEI. Filters were washed four times with ice cold hH₄R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

**Radioligand dissociation assay.** Cells used for radioligand binding studies were harvested 48h post transfection and homogenized in ice cold hH₄R binding buffer (50 mM Tris, pH 7.4). Cell membranes were incubated for 1 hour with 10-20 nM of [³H]histamine. The [³H]histamine was dissociated by addition of 10 μM of the selective H₄R antagonist JNJ 7777120 or 1 μM of CCL5 (RANTES). Samples were collected at different time intervals. Non dissociated radiolabel was harvested by filtration through GF/C filterplates. Filters were washed four times with ice cold hH₄R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

**NF-κB-luciferase reporter gene assay.** HEK 293T cells transiently co-transfected with 5 μg pNF-κB-Luc and 1 μg pcDEF-H₄R and 1 μg pcDEF3-US28 were seeded in 96-well whiteplates (Costar, Cambridge, MA) in culture medium and after 24 h incubated with ligands. After 48 h, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μl/well luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM MgCl₂, 0.78 μM Na₂H₄P₂O₇, 38.9 mM Tris, pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 μM dithiothreitol). After 30 min, luminescence was measured for 3 s/well in a Victor² 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences).

**[³H]inositol phosphate accumulation assay.** Cells were seeded in 24-well plates, and 24 h after transfection labelled overnight in inositol-free-medium supplemented with 1μCi/ml myo-[²-³H]inositol. Subsequently, the labeling medium was aspirated, cells were washed with PBS and pre-incubated for 10 min with Dulbecco’s modified Eagle’s medium containing 25 mM HEPES (pH 7.4), 20 mM LiCl₂. The pre-incubation medium was aspirated and cells were incubated in the same medium in the absence or presence of ligands. The incubation was stopped by aspiration of the medium and addition of cold 10 mM formic acid. After 90 min of incubation of ice, inositol phosphates were isolated by anion exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

**Analytical methods.** All radioligand binding data were evaluated by a non-linear least squares curve fitting program using Graphpad Prism® (Graphpad Software Inc, San Diego, CA). Protein concentrations were determined according to Bradford, using BSA as standard. All data are represented as mean ± S.E.M. from at least three independent experiments in triplicate. Statistical significance was determined by an ANOVA tuckey test (p<0.05 was considered statistically significant).
CHAPTER 7

Overproduction of the human histamine H4 receptor through the use of various expression systems

Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
Nijmegen Centre for Molecular Life Sciences, Department of Biochemistry, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands
BioXtal, Chemin des Croisettes 22, CH-1066 Epalinges, Switzerland
ABSTRACT

The human histamine H₄ receptor (hH₄R) is the fourth and latest discovered member of the histamine family of G protein coupled receptors (GPCRs). The H₄R has been suggested to play a role in e.g. inflammatory diseases like allergic rhinitis and rheumatoid arthritis. Recently, suggestions have been made for the H₄R to be involved in certain forms of cancer as well, showing the potential for the H₄R as interesting drug target. The rational design of new drugs strongly benefits from high resolution structural information of the target. However, to obtain this information, using biophysical techniques, large and pure amounts of the GPCR are required.

This study describes our attempts to overexpress the hH₄R using several different techniques, including viral expression systems and codon optimization of the H₄R. Both the Semliki Forest virus and the baculovirus expression system were employed to overexpress the H₄R. While highest H₄R expression was obtained with the baculovirus system we also found that epitope tagging of the H₄R could significantly enhance H₄R expression levels, especially hemagglutinin and c-myc epitope tags gave marked increases in H₄R expression. On the other hand codon optimization only resulted in a maximal 2-fold increase in H₄R expression. Combining several of the strategies used, could help to increase H₄R expression to start purification efforts and subsequently biophysical studies.

INTRODUCTION

G protein coupled receptors (GPCRs) form one of the largest family of drug targets for the pharmaceutical industry."
structure of this GPCR. Until today rhodopsin remains the only GPCR for which such a high resolution structure has been produced. However, for the other GPCRs, the use of computer models together with the protein sequence of the receptor has resulted in the formation of homology models i.e. computer models of GPCRs for which the protein sequence has been overlaid on the rhodopsin structure. These models can be improved with the data obtained from mutational studies. An additional option is to obtain structural data for the receptor target directly.

Besides the hydrophobic membranous nature of GPCRs, one of the main reasons why no other GPCRs have been crystallized so far is the difficulty to obtain large and pure enough amounts of receptor protein. Under physiological conditions most of the GPCRs are expressed at very low levels. Over the last decade several methods have been exploited to overexpress GPCRs. For example, *E.coli* and certain yeast strains like *Saccharomyces cerevisiae* and *P. pastoris* have been widely employed for this purpose. However, expression of GPCRs in non-mammalian cells suffer from the lack of post-translational modifications, which can affect the folding and transport of these proteins, and potentially GPCR functionality. The use of viral expression systems has facilitated large scale production of many GPCRs. The two most commonly used viral expression systems are the baculovirus and the Semliki Forest virus. Finally epitope tagging or codon optimization of GPCRs can also result in significant increases of receptor expression.

Six years ago a fourth receptor belonging to the family of histamine receptors was cloned. This histamine H4 receptor (H4R) is suggested to play a role in inflammatory diseases and might also be involved in rheumatoid arthritis and cancer, making the H4R an interesting new drug target. To facilitate the synthesis of high affinity and selective ligands targeting the H4R the availability of detailed structural information of the H4R binding pocket would be helpful. Such structural information can only be obtained with large quantities of H4R protein. Since the H4R is not endogenously expressed at high levels, recombinant methods have to be employed to enhance the expression level of the H4R.
Herein we report on our attempts to overexpress the human histamine H₄ receptor using several different strategies. We have employed both baculovirus and Semliki Forest virus as well as differentially epitope tagging and codon optimizing the hH₄R. In our hands higher levels of H₄R expression was obtained using the baculovirus expression system than by employing the Semliki Forest virus expression system. We found that codon optimization resulted only in a minor increase of H₄R expression, whereas the use of certain epitope tags resulted in a 10-fold increase in expression over wild-type H₄R expression.

RESULTS

Overexpression of the hH₄R through codon optimization and epitope tagging.
In 1999 Mirabekov et al. enhanced the expression of the CCR5 chemokine receptor by codon optimizing the coding region of the CCR5 gene. The codon optimized CCR5 showed 2-5 times enhanced expression compared to the wild-type receptor. Incubation with sodium butyrate further increased the expression of the CCR5. Codon optimization is considered to facilitate the transcription/translation machinery by removing rare codons from the gene of interest. To determine whether codon optimization of the hH₄R would help increase its expression level we developed a codon optimized hH₄R (hH₄coR). The gene for the H₄coR was constructed to predominantly use only the most abundant codon for each amino acid (Figure 7S). The following codons were used: alanine (GCC), arginine (AGA), asparagine (AAC), aspartate (GAC), cysteine (TGC), glutamate (GAG), glutamine (CAG), glycine (GGC), histidine (CAC), isoleucine (ATC), methionine (ATG), leucine (CTG), lysine (AAG), phenylalanine (TTC), proline (CCC), serine (AGC or TCC), threonine (ACC), tryptophan (TGG), tyrosine (TAC), valine (GTG) (Figure 7S). Expression of the hH₄coR in HEK293T cells (Figure 7.1A) or COS-7 (Figure 7.1B) resulted maximally in a 2-fold increase in the expression level of the wild type H₄R (0.2 and 1.5 pmol/mg protein in HEK 293T and COS-7, respectively).

Epitope tagging of a receptor can have a significant effect on receptor expression levels. To determine the effect of N-terminal epitope tags on the expression
level of the wild-type H₄R, we created several H₄R constructs; a hemagglutinin tagged H₄R (HA-H₄R), a FLAG tagged H₄R (FLAG-H₄R), a c-myc tagged H₄R (myc-H₄R) and an N-terminally c-myc tagged plus C-terminally histidine tagged H₄R (myc-H₄R-His₁₀). When transiently expressed in HEK 293T (Figure 7.1A) or COS-7 cells (Figure 7.1B) a 2- and 11-fold increase in expression level was observed for the HA- and myc-tagged H₄Rs compared to the wild-type H₄R in COS-7 and HEK 293T cells, respectively. The introduction of a FLAG epitope tag did not show a marked effect on H₄R expression level in COS-7 cells, while in HEK 293T cells a relatively small 3-fold increase was observed. A >10-fold increase was observed with the HA- and c-myc-tagged H₄Rs.

Figure 7.1. Recombinant expression of a codon optimized H₄R and differentially tagged H₄Rs in mammalian cells. HEK 293T (A) or COS-7 (B) cells were transiently transfected with either wild-type H₄R, codon optimized H₄R (H₄coR), FLAG-H₄R, HA-H₄R, c-myc-H₄R, or c-myc-H₄R, expression levels were determined by [³H]histamine displacement.

Another option to increase receptor expression levels is the addition of sodium butyrate in the growth medium. Sodium butyrate is a histone deacetylase inhibitor found to arrest growth and to induce differentiation in various cell types by modulating gene expression ⁴⁰⁴. For several GPCRs incubation of the cells with sodium butyrate has been shown to enhance receptor expression ⁴⁰²,⁴⁰⁵. To test whether sodium butyrate has a positive effect on H₄R expression levels we produced a CHO cell line stably expressing the H₄coR. However, 48 hour incubation
of stably transfected CHO-H4coR cells with 5 mM, 10 mM or 50 mM sodium butyrate did not have a positive effect on the expression of the H4R (Figure 7.2). Expression levels can be enhanced by increasing the number of receptors that are correctly folded on the plasma membrane. Pre-incubation with ligand may stabilize the receptor.\textsuperscript{406,407} Previously we have shown that incubation with the H2R inverse agonist ranitidine has a positive effect on H2R expression levels \textsuperscript{408}. However, incubation of stably transfected CHO-H4coR with the H4R inverse agonist thioperamide (10 \(\mu\)M) for 24 hours resulted in a significant decrease in the H4R expression level (2.5 pmol/mg protein vs 1.6 pmol/mg protein), while longer incubation (48 hours) resulted in an even stronger, 2-fold, reduction in H4R expression (Figure 7.2).

\textbf{Figure 7.2.} Effect of sodium butyrate and inverse agonist incubation on H4R expression in CHO cells, stably expressing the H4coR. CHO cells stably expressing H4coR were cultured in the presence or absence of the H4R inverse agonist thioperamide (10 \(\mu\)M, TP) for 24 and 48 hours or different concentrations of sodium butyrate (5, 10 or 20 mM) for 48 hours. Expression levels were determined by \(^{3}H\)histamine displacement.
Overexpression of the hH4R using Semliki Forest virus.

We have previously successfully employed the SFV viral expression system to increase rH2R expression levels in infected COS-7 cells [408]. One of the main advantages of the SFV over the baculovirus expression system (BEVS) is the ability to infect a variety of mammalian cells e.g. CHO, COS-7, BHK, HEK 293 [409]. To investigate the ability of the SFV expression system to facilitate high expression of the hH4R we constructed an N-terminally FLAG and C-terminally His10 tagged H4R (FLAG-H4R-His10) in the pSFVgenB vector. The epitope tags were used for eventual detection and purification of the receptor, respectively. To determine what the optimal time of infection is to reach maximal H4R expression for SFV infection, cells were infected with recombinant SFV and harvested at different time points. For H2R expressed in COS-7 cells using recombinant SFV, expression reached a plateau after 16 hours, which was sustained for at least 24 hours (Hoffmann et al., 2001). We infected CHO cells with SFV-H4R and harvested the cells either 24 hours (1 dpi) or 48 hours (2 dpi) after infection and H4R expression level were determined using [3H]histamine binding (Figure 7.3A). Compared to 1 dpi, a drastic decrease in H4R expression level was observed at 2 dpi (Figure 7.3A). Therefore, to determine expression levels in recombinant SFV-H4R infected in COS-7, BHK, HEK 293T or CHO cells were harvested 24 hours after infection. The expression level of H4R in BHK, HEK 293T and CHO cells were 2-fold (BHK) and 5-fold (HEK 293T and CHO) lower than that in SK-N-MC neuroblastoma cells stably expressing the H4R (Figure 7.3B). SFV-H4R infection in COS-7 cells resulted in H4R expression levels that were slightly higher than the expression in the SK-N-MC-H4R cell line (2.45 vs 1.8 pmol/mg protein).

To characterize the pharmacology of the H4R in infected CHO cells radioligand displacement assays were performed in which [3H]histamine was displaced by a selection of typical H4R ligands: the endogenous agonist histamine, the full agonist clobenpropit, the neutral antagonist JNJ7777120 and the inverse agonist thioperamide (Figure 7.3C). The obtained pK_i-values of these compounds for the FLAG-H4R-His10 in infected CHO cells were comparable to the values obtained for the H4R in stable transfected SK-N-MC cells (Table 7.1).
Figure 7.3. Expression of FLAG-H₄R-His₁₀ in mammalian cell lines infected with recombinant Semliki Forest virus. A. Infected CHO cells were harvested one or two days after infection (dpi) with recombinant Semliki Forest virus. B, COS-7, BHK, HEK 293T and CHO cells were infected with recombinant SFV. As control SK-N-MC neuroblastoma cells stably expressing H₄Rs were used. H₄R expression was determined by [³H]histamine displacement. C, Displacement of [³H]histamine by typical H₄R ligands on Semliki Forest virus infected CHO cells recombinantly expressing FLAG-H₄R-His₁₀.
Table 7.1. Characterization FLAG-H4R-His10 in Semliki Forest virus infected CHO cells or baculovirus infected Spodoptera frugiperda Sf9 cells. As control SK-N-MC neuroblastoma cells stably expressing the wild-type H4R were characterized. The K_0 values for histamine in nM, and the pK_i values of histamine and thioperamide for the H4R constructs used in the experiments as determined by [3H]histamine saturation and displacement binding assays. The values are expressed as mean ± SEM of at least three separate experiments performed in triplicate.

<table>
<thead>
<tr>
<th>FLAG-H4R-His10</th>
<th>[3H]histamine</th>
<th>pK_i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_0 (nM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histamine</td>
<td></td>
</tr>
<tr>
<td>CHO (SFV infected)</td>
<td>24 ± 22</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>Sf9 (baculovirus infected)</td>
<td>153 ± 17*</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>125.9**</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>SK-N-MC-H4R</td>
<td>21 ± 2</td>
<td>7.6 ± 0.1</td>
</tr>
</tbody>
</table>

* determined by homologous displacement
** determined by saturation binding (n=1)

Overexpression of the hH4R using baculovirus.

Currently, we are successfully employing the BEVS to produce large amount of the hH1R. The BEVS is frequently used to study GPCRs, as the baculovirus infects insect cells naturally devoid of endogenous mammalian GPCRs. Although these insect cells do not have the same post translational modification mechanism of mammalian cells, it resembles the mammalian system better than yeast or E.coli expression system. However, the generation of recombinant baculovirus can be a difficult and tedious job. We have employed the FastBac™ system to obtain recombinant baculovirus containing the gene encoding the FLAG-H4R-His10 relatively quickly. When infecting Spodoptera frugiperda (Sf9) cells expression of the hH4R was measured using [3H]histamine radioligand binding. Generally, baculovirus infected Sf9 cells reach maximal expression of the recombinant gene around 4-6 dpi. Baculovirus-H4R infected Sf9 cells were harvest at 3, 4, 5 and 6 days after infection (3-6 dpi) and H4R expression level were determined using [3H]histamine binding (Figure 7.4A). H4R expression was relatively low at 3 and 4 dpi, but roughly doubled after 5 days, and remained stable at 6 dpi (Figure 7.4A).
Figure 7.4. Expression of FLAG-H4R-His10 in Sf9 insect cells infected with recombinant baculovirus. A, Infected Sf9 cells, grown in TNM-FH medium, were harvested at different time points, starting from 3 dpi till 6 dpi with recombinant baculovirus. B, Infected Sf9 cells were grown in TNM-FH medium and harvested 5 days after infection (dpi). C, Infected Sf9 cells were grown in either TNM-FH medium (black bar) or serum free Xpress medium. Infected cells grown in TNM-FH medium were incubated in the presence (closed bars) or absence (open bars) of 20 μM thioperamide (TP) and harvested at 4 dpi. H4R expression levels were determined by [3H]histamine displacement. D, Displacement of [3H]histamine by typical H4R ligands on baculovirus infected Sf9 cells recombinantly expressing FLAG-H4R-His10.
Using an M.O.I of 1 and harvesting at 5 dpi expression of the FLAG-H$_4$R-His$_{10}$ maximally reached 14 pmol/mg protein (Figure 7.4B). To compare the effect of expression medium, Sf9 cells grown in either serum rich TNM-FH medium or serum free insect Xpress medium, were infected with baculovirus (M.O.I. = 1) and were harvested at 4 dpi. Expression levels were significantly higher in Sf9 cells grown in TNM-FH medium than in insect Xpress medium (Figure 7.4C). H$_4$R expression did not benefit from incubation with 20 μM of the H$_4$R inverse agonist thioperamide during culture, which, by stabilizing the receptor, could have assisted in inducing and maintaining proper folding and subsequent transport of the H$_4$R to the plasma membrane (figure 7.4C).

The pharmacology of the H$_4$R in infected Sf9 cells was characterized by radioligand displacement assays in which [³H]histamine was displaced by histamine, clobenpropit, JNJ7777120 and thioperamide (Figure 7.4D). The pharmacology of the FLAG-H$_4$R-His$_{10}$ in baculovirus infected Sf9 insect cells differed from that of H$_4$Rs stably expressed in SK-N-MC neuroblastoma cells as the affinity of several tested H$_4$R ligands showed an almost ten fold drop in pK$_i$-values compared to the H$_4$R in mammalian cells (Table 7.1).

**DISCUSSION**

Over 1% of the human genome is encoded by G protein-coupled receptors (GPCRs). These genes give rise to the existence of over 1000 GPCRs. Many GPCRs are involved in diseases and as such make for interesting targets for the pharmaceutical industry. It is estimated that approximately 50% of available therapeutic drugs target GPCRs. While more money is spend every year in research, development and production of these drugs, fewer and fewer new drugs are successfully brought to the market. Part of this growing problem lies in the difficulty to find more selective and high affinity compounds for existing targets. In most cases high throughput screenings are employed to find potential leads for new or existing drug targets. In the end these leads still require optimization to increase their selectivity and affinity. This step is for the larger part trial and error based, as detailed information on the receptor binding pocket is scarce. Up till today the only high resolution structure that has been resolved for a GPCR comes...
from X-ray crystallography of rhodopsin. As for other membrane proteins it is very difficult to obtain good crystals of GPCRs for X-ray crystallography. Considering that under physiological conditions the expression of GPCRs is relatively low, an important obstacle is the requirement of large amounts of pure protein. Over the last decades several methods have been developed to overexpress GPCRs. GPCRs have been expressed in bacteria (E.coli), yeast (Saccharomyces cerevisiae, P. pastoris) or in insect cells using baculovirus infection. Another viral expression system which has been broadly used to infect mammalian cells is the Semliki Forest virus. All of these methods have their advantages and disadvantages, but none of these methods have so far successfully produced new high resolution structures of GPCRs. To overcome the problems encountered when pursuing large scale production and purification of GPCRs several national and international networks have been established. Examples include Joint Center for Structural Genomics (JCSG), E-Mep and Membrane Protein Network (MePNet).

In recent years we have had success in overexpressing the human histamine H\textsubscript{2} receptor using recombinant Semliki Forest virus and the human histamine H\textsubscript{1} receptor using recombinant baculovirus. Six years ago a new member of the histamine receptor family was identified and cloned. The H\textsubscript{4}R is expressed predominantly on leukocytes and mast cells and has a potential role in inflammatory diseases like allergic rhinitis and rheumatoid arthritis. Recently the H\textsubscript{4}R receptor was also linked to a possible role in colon cancer, making this receptor an interesting target for the pharmaceutical industry.

To facilitate drug design we attempt to obtain structural information of the H\textsubscript{4}R through biophysical techniques like NMR, MS or FT-IR. The first step in this process is the large scale production and subsequent purification of the H\textsubscript{4}R protein. To enable purification of the receptor often a short epitope tag is introduced N- or C-terminal of the receptor gene. In some cases the addition of such a tag has no influence on receptor expression level. For example the introduction of an N-terminal c-myc tag to the serotonin 5-HT\textsubscript{1B} receptor did not result in a significant increase of the expression level using recombinant baculovirus. On the other hand double tagging (His\textsubscript{6}-FLAG) of the adenosine A\textsubscript{1}...
and in particular the A2A receptors showed marked amplification of receptor expression levels compared to wild-type receptors. To investigate the influence of epitope tagging on H4R expression level a broad range of differentially tagged H4Rs were examined. In the case of the H4R the most significant increase (3-10 fold) in expression levels were obtained using either c-myc- or HA-epitope tags. A similar positive influence of the HA-epitope tag was apparent for the H1R (unpublished observation).

Previous studies on the CCR5 chemokine and the human herpesvirus encoded U51 receptors have shown that codon optimization can enhance receptor expression levels from 2-5 fold (CCR5) to 10-100 fold. However, codon optimization of the H4R gene did not result in a marked increase of H4R expression. Maximally a 2-fold increase was observed in transiently transfected HEK 293T cells. Apparently the codon usage of the H4R receptor is not the rate limiting step in the production of the H4R protein.

We have previously demonstrated that the use of viral expression systems can be a valuable tool in the large scale production of histamine receptors. Infection of COS-7 cells with recombinant SFV-rH2R resulted in H2R expression levels up to 50 pmol/mg protein. Also expression levels of other GPCRs have been successfully amplified using SFV. Expression levels over 30 pmol/mg protein have been obtained for the metabotropic glutamate R1 receptor, the adrenergic α2BR and the human neurokinin receptor NK1R. However, infection of recombinant SFV-FLAG-H4R-His10 in a set of different mammalian cell lines did not result in expression levels exceeding that of the wild-type H4R in stably expressing SK-N-MC neuroblastoma cells (2.5 pmol/mg protein). The use of different epitope tags e.g. c-myc or hemagglutinin could help improve expression levels as was shown in transiently transfected cells (this manuscript).

Besides SFV, the use of baculovirus expression vector system (BEVS) has been widely employed to express (human) GPCRs in insect cells. We have successfully used the BEVS to produce and purify large amounts of the hH1R. Infection of SF9 cells with recombinant baculovirus containing the FLAG-H4R-His10 resulted in H4R expression levels maximally ranging from 9-14 pmol/mg protein. However, the pharmacology of the H4R differed from the H4R expressed in mammalian cells. A
possible explanation for the decreased binding affinity of H₄R ligands towards the FLAG-H₄R-His₁₀ expressed in Sf9 cells could be that the tags have a negative effect on the binding pharmacology of the H₄R. However, the same double tagged receptor did not affect the binding of ligands when expressed in mammalian cells using the SFV. Therefore it is more likely that the low abundance of native G-proteins in insect cells is the reason for the observed decrease in affinity. The effect of G-proteins would be more pronounced when using an agonist radioligand, as is the case for the H₄R binding ([³H]histamine). In the present experimental set up a FLAG-H₄R-His₁₀ was used. However, it turned out that the FLAG-epitope tag has the least beneficial effect on H₄R expression levels. It would therefore be of interest to generate a recombinant baculovirus containing c-myc or HA epitope tagged H₄R. Also the H₄R was placed behind the p10 promoter, whereas high expression for e.g. the hH₁R and the turkey β-adrenergic receptor were obtained when the gene was under control of the polyhedron promoter. Although the use of both SFV and BEVS is widely spread, these methods have not yet resulted in the production of purified receptors suitable for high resolution structure elucidation. Therefore it is important to also consider different approaches. A technique that does not require any host cell is the cell free expression system. This method has recently been employed to successfully produce milligrams of human β₂-adrenergic receptor protein. While no post-translational modifications are applied to the receptor, production of protein is much faster than using viral expression systems and also the produced protein is already relatively pure compared to proteins produced in cells. Altogether this makes the cell free expression system potentially valuable for large scale production of GPCRs.

In summary, we have investigated several different methods to overexpress the human H₄R in a various range of different cell lines. We found that epitope tagging the H₄R with an N-terminal c-myc or a hemagglutinin HA tag resulted in significant increase (up to 10-fold) of H₄R expression. Codon optimization of the H₄R or introduction of an N-terminal FLAG tag did not have a marked effect on H₄R expression. Whereas for the H₂R, SFV gave higher expression than the BEVS, for the hH₄R highest expression levels (up to 14 pmol/mg protein) were obtained using baculovirus. The obtained knowledge can be used to choose and
optimize the best technique to increase H₄R expression levels high enough to facilitate large scale production of H₄R protein to continue with GPCR purification and potentially structural studies like MS, NMR, FT-IR or X-ray crystallography.

**MATERIALS AND METHODS**

**Materials.** Bovin serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine(2-[4-imidazolyl]ethylamine hydrochloride), polyethyleneimine Leupeptin and TNM-FH insect medium was purchased from Sigma (USA). Cellfectin, pFastBac_Dual vector and DH10Bac competent cells were from Invitrogen (Breda, The Netherlands). Calf serum (Integro BV, Dieren, The Netherlands). Insect-Xpress medium and Sea plaque agarose was from Cambrex (Walkersville, MD, USA). Cell culture media, penicillin, and streptomycin were obtained from Life technologies (Merelbeke, Belgium). Cell culture plastics were from Greiner Bio-one (Wemmel, Belgium). Tris was from AppliChem (Darmstadt, Germany). [³H]histamine (12.40 and 18.10 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Geneticin (G418), kanamycin, gentamycin and tetracyclin were purchased from Duchefa (The Netherlands). Oligonucleotides were purchased from Isogen Biocience (Maarsen, The Netherlands). PfuTurbo® DNA polymerase was purchased from Stratagene (La Jolla, CA). Restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). Thioperamide fumarate, clobenpropit dihydrochloride and JNJ 7777120, were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands. Gift of the expression vector pcDEF₃ (Dr. J Langer, 221 is greatly acknowledged. Wild type human H₄R in pcDNA3.1 was purchased from Guthrie cDNA resource center (Sayre,PA). The vector was subcloned into the pcDEF₃ using BamHI/XbaI sites. The codon optimized H₄R was purchased from GENEART GmbH (Regensburg, Germany) and supplied in a pPCR-script AMP vector (Figure 7S). The H₄coR gene was subclone into the pcDEF₃ expression vector using BamHI/XbaI sites.

**Cell culture and transfection of mammalian cells.** COS-7 African green monkey kidney, Chinese hamster ovarian (CHO) and human embryonic kidney (HEK 293T) cells were maintained at 37°C humidified in 5% CO₂/95% air atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 50 IU/ml of penicillin and 50 μg/ml streptomycin and grown in 100 mm dishes. Cells were transiently transfected using the DEAE-dextran method as previously described 85. The total amount of DNA transfected was maintained constant by addition of pcDEF₃.

**Cell culture of Spodoptera frugiperda (Sf9) cells.** Sf9 cells were cultured as monolayers and maintained at 27°C in TNM-FH medium supplemented with 10% (v/v) FCS, 50 IU/ml penicillin and 50 μg/ml streptomycin or insect Xpress supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin.
[³H]Histamine binding studies. Cells used for radioligand binding studies were harvested 48h post transfection and homogenized in ice cold H₄R binding buffer (50 mM tris, pH 7.4). For saturation isotherms cell membrane homogenates were incubated at 37°C for 60 minutes with 0-125 nM [³H]histamine in a total assay volume of 200 μl. Non-specific binding was determined by incubation in the presence of 10 μM JNJ 7777120. For competition binding assays the cell homogenates were incubated at 37°C for 60 minutes with 0.1-10.000 nM ligand in the presence of ~15 nM [³H]histamine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice cold H₄R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filterplates that had been treated with 0.3% polyethyleneimine. Filters were washed four times with H₄R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

Construction of epitope-tagged H₄Rs. The construction of FLAG-H₄R and HA-H₄R and c-myc-H₄R-His₁₀ has been described before. An N-terminally c-myc (EQKLISEEDL) epitope tagged H₄ receptor was created by PCR in two steps. The c-myc tag was amplified by PCR using a pcDEF3-Myc-H₄ vector (kind gift of Steve Hill) as template with a 3’ NheI site using the following sense 5’-GGGTGGAGACTGAAGTTAGGCC-3’ and antisense primer 5’-GTGCTAGCAGTCTCTGGAG-3’. The fragment was directly cloned to the pCRII-Topo vector (pCRII-topo-myc). The H₄ gene was amplified without start codon and containing a 5’ Nhel restriction site using the following sense 5’-CCGCTAGCCAGATACTAATAGCAC-3’ and anti sense primer 5’-TCTTTAAGAAGATACTGACC-3’. The gene was directly cloned to the pcDNA3.1/ V5-His-Topo vector. The H₄ gene was subsequently cloned in frame using Nhel/NsiI into the pCRII-topo-myc vector (pCRII-topo-myc-H₄). The resulting myc-H₄ gene was subsequently cloned into the pcDEF3 expression vector using the BamHI/XbaI sites. The construct was fully sequenced before its expression and analysis.

Generation of stable cell lines. CHO cells were transfected with pcDEF3-H₄coR, pcDEF3-H₄-eYFP or pcDEF3-myc-H₄R-His₁₀ using polyfect (Qiagen Benelux, Venlo The Netherlands) as described by the manufactures protocol. In short CHO cells were transfected with 4 μg of DNA using polyfect and splitted 1:100 in culture medium containing 1 mg/ml G418. Single colonies of cells were picked and H₄R expression was verified through radioligand binding to select clones highly expressing the H₄R.

Generation of recombinant Semliki Forest virus particles. A N-terminally tagged FLAG and C-terminally tagged His₁₀ H₄ gene in a suitable vector for creation of Semliki Forest virus was created by PCR in two steps. The H₄ gene was amplified from the pcDEF3-H₄R vector by PCR without start and stop codon with a 5’BamHI site and a 3’ SpeI site using the following sense 5’-CCGGATCCCGATCCAGTATAGCACATCAA-3’ and anti sense primer 5’-CCGCGGCCGC ACTAGTAAAGATGACTGACCC-3’. The gene was directly cloned into the pCRII-Topo vector. The H₄ gene was subsequently cloned in frame from the pCRII-topo-vector using BamHI/Spel sites in the pSFV2genB vector. The construct was fully sequenced before its expression and analysis. pSFV2genB–FLAG-H₄R-His₁₀ and pSFV–helper2 plasmids were linearised with SapI and SpeI, respectively, and were purified by phenol/chloroform extraction prior to in vitro transcription. RNA was
Overexpression of H4R

synthesised in vitro driven from the bacterial SP6 promoter. 2.5 μg of linearised plasmids were transcribed with 7 U SP6 RNA polymerase (Amersham Pharmacia Biotech) in a buffer containing 40 mM HEPES (pH 7.4), 6 mM MgOAc, 2 mM spermidine, 1 mM of ATP, CTP and UTP, 0.5 mM GTP, 1 mM m7G(5’)ppp(5’)G (CAP; Amersham Pharmacia Biotech), 1.5 U RNAsin (Roche) and 5 mM dithiothreitol in a final volume of 50 μl for 1 h at 37 °C. BHK-21 cells from a semi confluent 168 cm² flask were detached with Versene/Trypsine solution. Cells were collected by centrifugation for 5 min at 1500 rpm and washed twice with 10 ml PBS. Cells were resuspended in 2.4 ml PBS prior to electroporation with the in vitro synthesised RNA. 400 μl of BHK-21 cell suspension was transferred to a Biorad Gene pulser cuvette (Biorad, 165–2086) together with 50 μl of transcribed pSFV2genB–FLAG-H4R-His10 RNA and 25 μl of transcribed pSFV–helper2 RNA. Cells were electroporated twice in a Biorad Gene pulser (settings: 25 μF, 1500 V, 10Ω). Following electroporation cells were immediately resuspended in growth medium and seeded in 25-cm² flasks. 24 h after electroporation, the medium from the cells was collected, passed through a 0.22-μm filter. Viral particles were stored at -80 °C.

Infection of mammalian cell with SFV Prior to infection the recombinant virus was activated by chymotrypsin treatment. 500 μg Chymotrypsin (Roche Biochemicals) was added per 10 ml of virus suspension and incubated for 15 min at room temperature. Subsequently, the chymotrypsin was inactivated by the addition of 250 μl aprotinin (10 mg/ml, Sigma). Infection of mammalian cells with the activated viral particles was similar for all used cell lines. Cells were grown to 80% confluency, the medium was aspirated and cells were washed once with PBS. Diluted viral suspensions were added in a small volume, just enough to cover the cells. In most experiments, the original viral suspension was diluted 10-fold. This did not result in a significant loss of expression. Cells were incubated with the virus suspension at 37 °C for 1 h, after which appropriate culture medium was added and the cells were cultured for 24–48 h.

Construction and generation of recombinant baculovirus. To produce recombinant baculovirus containing the H4R an N-terminally tagged FLAG and C-terminally tagged His10 H4 gene was cloned from the pSFV2genB-FLAG-H4R-His10 behind the p10 promoter of the pFastBac_DUAL vector using NcoI/NheI restriction sites. The construct was fully sequenced before its expression and analysis. The pFastBac_DUAL-FLAG-H4R-His10 was transformed into DH10BAC cells using heat-shock. To identify correctly transformed DH10BAC cells, the cells were plated out on LB plates containing ampicillin, kanamycin, tetracyclin and gentamycin resistance together with blue/white screening using X-gal en IPTG. After isolation of Bacmid DNA, Sf9 cells were transfected with cellfectin and individual virus particles were isolated via a plaque assay. The recombinant virus was amplified by infecting Sf9 cells with the individual selected plaques, which had been dissolved in TNM-FH expression medium for 7 days. The titer of the virus was determined via plaques assay and a WST-1 colorimetric assay. Finally, amplified virus was used to infect Sf9 cells, which could be harvest in general 3-5 days after infection (dpi).
Analytical methods. Binding data were evaluated by a non-linear least squares curve fitting program using Graphpad Prism® (Graphpad Software Inc, San Diego, CA). Protein concentrations were determined according to Bradford\textsuperscript{[227]}, using BSA as standard. All data are represented as mean ± S.E.M. from at least three independent experiments in triplicate. Statistical significance was determined by a Students unpaired t-test (p<0.05 was considered statistically significant).

ATG CCC GAC ACC AAC AGC ACC ATC AAC CTG AGC CTG AGC
ACC AGA GTG ACC CTG GCC TTC TTC ATG AGC CTG GTG GCC
TTC GCC ATC ATG CTG GGC AAT GCC CTG GTG ATC CTG GCC
TTT GGT GGT GAC AAC AAC CTG CGG CAC CGG AGC AGC TAC
TTT TTC CTG AAC CTG GCC ATC AGC GAC TTT TTT GTG GGC
GCG ATC AGC ATC CCC CTG TAC ATC CCC CAC ACC CTG TTT
GAG TGG GAC TTC GGC AAC GAG ATC TGC GTG TTT TGG CTG
ACC ACC GAC TAC CTG CTG TGC ACC GCC AGC GTG TAC AAC
ATC GTG CTG ATC TCC TAC GAC CGC TAC CTG AGC GTG TCC
AAC GCC GTG TCC TAC AGA ACC CAG CAC ACC GCC GTG CTG
AAG ATC GTG ACC CTG ATG GTG GCT GTG TGG GTG CTG GCC
TTC CTG GTG AAC GCC CCC ATG ATC CTG GTG TCC GAG AGC
TGG AAG GAC GAG GCC AGC GAG TGC GAG CCC GCC TTC TTT
AGC GAG TGG TAC ATC CTG GCC ATT ACC AGC TTC CTG GAG
TTT GTG ATC CCC GTG ATT CTG GTG GCC TAC TTT AAC ATG
AAC ATC TAC TGG AGC CTG TGG AAG AGA GAC CAC CTG AGC
CGG TGC CAG AGC CAC CCT GCC CTG ACC GCC GTG TCC AGC
AAC ATC TGC GCC CAC AGC TTC AGA GCC AGG CTG AGC AGC
AGA AGA AGC CTG AGC GCC AGC ACC GAA GTG CCC GCC AGC
TTC CAG AGC GAG CGG CAG AGA AGA AAG AGC AGC CTG ATG
TTC AGC AGC CCG ACC AAG ATG AAC AGC AAC ACC ATC GCC
AGC AAG ATG GCC AGC TTC ATG CAG AGC GAC AGC GTG GCC
CTG CAC CAG AGA GAG CAC GTG GAG CTG CTG AGA GCC AGG
AGA CTG GCC AAG AGC CTG GCC ATC CTG GTG GGA GTG TTT
GCC GTG GTG TGG GCC CCC TAC AGC CTG TTC ACC ATC GTG
CTG TCC TTC TAC AGC GCC ACC GCC CCC AAG TCT GTG
TGG TAC AGG ATC GCC TTC TGG CTG CAG TGG TTC AAC AGC
TTC GTG AAC CCC CTG CTG TAC CTC TG TGC CAG AAG AGA
TTC CAG AAG GCC TTC CTG AAG ATC TTC TGC ATC AAG AAG
CAG CCC CTG CCC AGC CAG CAC AGC AGA AGC GTG TCC AGC

Figure 7S: DNA sequence of the codon optimized human histamine H\textsubscript{4} receptor
CHAPTER 8

Discussion and conclusion

Van Rijn RM, Bakker RA, Leurs R
Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry,
Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
Over 1% of the humane genome is encoded by G protein coupled receptors (GPCRs)\(^1\). These receptors bind a large array of first messenger resulting in the modulation of a large diversity of intracellular signals, and their involvement in many physiological processes\(^{419}\). Histamine, an endogenous amine, acts on a subfamily of these GPCRs, the histamine receptors, causing (patho)physiological effects such as anaphylaxis and gastric acid release\(^79\). Over the years several therapeutics have been developed targeting the histamine H\(_1\) and H\(_2\) receptors (H\(_1\)R and H\(_2\)R)\(^2\), while therapeutics targeting the H\(_3\)R, are on the verge of entering clinical trials\(^{127,143}\).

The existence of an additional histamine receptor present on eosinophils was already predicted in 1975\(^{146}\). In 1994 Raible and co-workers showed that the pharmacology of this receptor differed from the three histamine receptors, known at the time\(^{147}\). Finally, the cloning of the H\(_4\)R in 1999\(^{123}\) facilitated the identification of this fourth receptor belonging to the histaminergic class of GPCRs at a molecular level a year later\(^{148-153}\). The work described within this thesis focuses on providing a more detailed characterization of this H\(_4\)R. At the time of the start of this Ph.D. project in 2002 only a few papers, besides the original cloning papers, had been published and little was known about the pharmacology of the H\(_4\)R. Based on the selective expression pattern of the H\(_4\)R in cells of the hematopoietic lineage, such as leukocytes and mast cells\(^{150-152}\), the receptor was suggested to play a role in inflammatory diseases already early on\(^{148,164,168}\). This idea was supported further by the discovery that the activation of the H\(_4\)R results in chemotaxis of eosinophils\(^{165,168}\), mast cells\(^{164}\), and monocyte-derived dendritic cells\(^{161}\). Additionally, release of several interleukins, which are important mediators in inflammation\(^{367}\) is mediated by the H\(_4\)R\(^{118,161,181,184}\). The link between the H\(_4\)R with allergic reactions was established both in mice\(^{179,184,186,187}\) and human\(^{163}\). However, additional roles for the H\(_4\)R were also identified. The H\(_4\)R may play a role in rheumatoid arthritis\(^{168}\), colon cancer\(^{189,190}\), and breast cancer\(^{191}\).

One of the reasons why it has been difficult to determine the physiological role of the H\(_4\)R is the lack of available selective ligands. Since the H\(_4\)R shares relatively high homology with the H\(_3\)R (58% within the TM domains\(^{149}\)) most of the available ligands have comparable affinity for the H\(_4\)R as for the H\(_3\)R. Often this problem
could be avoided as the expression pattern of the H₄R and the H₃R, expressed predominantly in the brain, hardly overlaps. Another option was the use of the partial agonist H₄R clobenpropit, which behaves as inverse agonist on the H₃R. The development of the selective H₄R antagonist JNJ 7777120 aided many researchers in their study of H₄R pharmacology. In our search to try and identify analogues of JNJ 7777120, that could possibly have even higher selectivity, we identified the benzimidazole analog VUF6002, a H₄R antagonist which showed comparable selectivity as JNJ 7777120. Besides a selective H₄R antagonist, the availability of a selective H₄R agonist would be very useful to determine the involvement of the H₄R in a certain pathway or disease, by mimicking the actions of histamine. Therefore we decided to screen a specific set of histaminergic compounds. This screen revealed 4-methylhistamine, previously characterized as moderate H₂R agonist, as high affinity (>100-fold over H₁R, H₂R and H₃R) H₄R agonist. The screen also yielded the identification of VUF 4742, a burimamide analogue, as second inverse agonist for the H₄R, with thioperamide being the first one. Synthesis efforts performed by other research groups confirmed VUF6002 and 4-methylhistamine as H₄R agonists. Further screening of clozapine and dimaprit analogues within our group have resulted in the discovery of other potent H₄R agonists such as (E)-7-chloro-11-(4-methylpiperazin-1-yl)dibenzo[b,f][1,4]oxazepine and VUF 8430. A high affinity H₄R ligand could also potentially be useful as radioligand. We have investigated three different radioligands for the H₄R: [³H]histamine, [³H]JNJ 7777120 and [¹²⁵I]iodophenpropit. All three ligands have their drawbacks. The most widely used radioligand is tritiated histamine. However, histamine does not have a very high affinity (~10 nM) and is not selective. While [³H]JNJ 7777120 is more selective, the affinity is not very different from histamine, and at the moment it is not commercially available. [¹²⁵I]iodophenpropit exhibits the highest affinity for the H₄R compared to histamine and JNJ 7777120. [¹²⁵I]iodine is an advantage for in situ radioligand binding and autoradiography assays. However, iodophenpropit is not selective for the H₄R as it binds with similar affinity to the H₃R. Additionally, [¹²⁵I]iodophenpropit suffers from high levels of aspecific binding. To study in more detail the suggested presence of the H₄R in brain the availability of a selective high affinity iodinated H₄R
radioligand, which can be iodinated would be very helpful. Another options would be the development of a high affinity, selective fluorescent H4R ligand, as have been created for the H3R already\textsuperscript{422} and to a lesser extent the H2R\textsuperscript{274}.

**Homo-oligomerization of the human histamine H4R**

The ability of monomeric proteins to form larger multimeric complexes has been shown for several classes of proteins, such as enzymes, ion channels and transcription factors\textsuperscript{258,259}. Additionally, several peptidergic ligands targeting GPCRs, such as chemokines, are also known to form oligomeric structures\textsuperscript{423}. However, until the early 90’s the general view was that GPCRs act as monomers interacting with the G-protein in a 1:1 stoichiometry. From that time more and more evidence emerged supporting the hypothesis that GPCRs exist and function as oligomers\textsuperscript{30,39,198,207}. From 2000 and onwards the use of biophysical techniques such as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) to study GPCR oligomerization has generated compelling evidence\textsuperscript{40}. Together with the finding that some receptors, such as GABAB and taste receptors need to form oligomers in order to function\textsuperscript{41-43,45,46}, this has eventually led to the general acceptation that GPCRs can also exist as oligomers.

In our study on the oligomerization of the H4R we employed both biochemical (co-immunoprecipitation, cross-linking) as well as biophysical assays (BRET, time-resolved FRET). The use of a selective H4R antibody allowed for the detection of H4R in native tissue (human T-lymphocytes and human spleen lysates). It appears that in human T-lymphocytes the H4R is predominantly present as a dimeric entity\textsuperscript{234}. The existence of H1R-H4R hetero-oligomers was dismissed, based on the finding that at expression levels, considered to be physiologically relevant, no H1R-H4R hetero-oligomers were detected in both the BRET and tr-FRET assays\textsuperscript{234}. The H1R was chosen as this receptor is co-expressed in several different cells also expressing H4R\textsuperscript{150,202}. Also both receptors have been shown to play a role in inflammatory diseases, such as allergy\textsuperscript{200,201}.

From a homology point of view it would have been logical to investigate hetero-oligomerization between the H3R and the H4R. Since the H3R is expressed...
predominantly in brain, while the H₄R is expressed almost exclusively in leukocytes and mast cells, the physiological relevance of H₃R-H₄R would be difficult to prove. However, recent studies showed that the H₃R is expressed together with the H₄R in human nasal mucosa ¹⁶⁰. Recently, using the H₄R antibody, the H₄R protein was identified in brain as well ⁴²¹. These findings make the study of H₃R-H₄R potentially interesting. The study of H₂R-H₄R hetero-oligomers would also be appealing as the H₄R can be found co-localized with the H₂R ⁴²⁴. Additionally H₂R and H₄R control histamine-induced IL16 release from human CD8⁺ T cells ¹¹⁸. Moreover, histamine was shown to exert pro-proliferative and pro-angiogenic effects via H₂R/H₄R receptor activation ¹⁹⁰.

The use of computer models is often applied to assist rational drug design. At the moment these computer models are based on the monomeric structure of rhodopsin. To evaluate the effect of dimeric GPCRs on the binding pocket and ligand binding to dimeric receptors, it would be very helpful and scientifically interesting to try and create a computer model, that would represent dimeric GPCRs. Very recently a first attempt to model GPCR dimers and trimers was published ⁴²⁵. Computer simulation might already predict what effect the binding of a ligand to one receptor has on the binding pocket of the other receptor. To make good predictions much more detailed information on the dimerization interface is however required. A good example of the type of data required is the study performed on the dopamine D₂ receptors by Guo and co-workers. In these studies amino-acids within TM IV were exchanged for cysteine residues and the importance of each amino-acid in the dimer interface was determined by cross linking ²⁴⁴,²⁹⁸

Identification of differentially spliced H₄R isoforms

Currently twenty splice variants have been identified for the human H₃R and ten for the rat H₃R ¹²⁴. Since the gene structure of the H₃R is very similar to the H₄R (both genes contain at least three introns ¹²⁵,¹²⁶) it was of interest to determine if the hH₄R gene can also be alternatively spliced. Using an RT-PCR strategy on purified eosinophils and mast cells we were able to identify two H₄R splice variants (H₄R(302) and H₄R(67)). The existence of other splice variants however, should not
be excluded. In March 2003, Merck described two H₄R isoforms, the H₄bR and the H₄cR in a patent (WO 03/020907 A2). In both the sequences of the H₄bR and H₄cR one or two additional nucleotides emerge, which can not be explained based on the genomic arrangement of the H₄R. In the gene of the H₄bR splice variant two extra cytosines emerge (C¹⁹⁷T¹⁹⁸C¹⁹⁹), instead of T³⁶¹ in the full length H₄R. In the gene of H₄cR an extra nucleotide (C³⁵⁷) is found. In both cases introduction of these additional nucleotides keeps the gene in the “original” reading frame of the full length H₄R. It is likely that the added nucleotides result from a sequencing error. This assumption is strengthened by the fact that the H₄bR is identical to the H₄R(67) isoform if the two additional nucleotides would be omitted. However, the H₄cR differs from the H₄R(302). Removal of the additional nucleotides from the H₄cR would result in the receptor as depicted in Figure 8.1 (from here on referred to as putative H₄cR). This would suggest that at least one more H₄R splice variant would exist. In contrast with the H₄R(302) and H₄R(67), this H₄cR would retain the aspartate residue, D⁹⁴ (3.32), presumed to be crucial for histamine binding, as well as for the high affinity binding of other known orthosteric H₄R ligands. Moreover, unlike the H₄R(302) and H₄R(67), the H₄cR retains a topology in which the C-terminal tail is intracellular. The presence of a fourth exon located behind the putative stop codon of the full length H₃R(445) resulted in the existence of additional H₃R isoforms. In our search we did not investigate the occurrence of alternative splicing in the 3’UTR of the full length H₄R Therefore, it is of interest to continue cloning efforts to identify other H₄R splice variants. Furthermore it can be interesting to construct the putative H₄cR and characterize this potential H₄R splice variant. The presence of the aspartate residue D⁹⁴ (3.32) and an intracellular C-tail, could potentially mean that unlike the H₄R(302) and H₄R(67), the H₄cR is able to bind ligands and signal by itself, although the fact that the receptor consists of only 3 TM domains may decrease the likeliness for this to be true.
Figure 8.1: Sequence alignment hydrophobicity plot of putative H₄CR. A. Amino acid sequence alignment between the hH₄R and putative H₄CR. The putative H₄CR has a deletion between TMIII and TMV and reaches a premature stop codon due to a frame shift after being alternatively spliced. B. Predicted topology of the putative H₄CR as determined by a hydrophobicity plot (http://www.cbs.dtu.dk/services/TMHMM/). The putative H₄CR is predicted to consist of three TM domains and retains its intracellular C-terminal tail.
Functionality of H₄R splice variants

It has been shown for many splice variants including the rH₃RDEF isoforms that non-7TM receptor isoforms can influence full length receptor trafficking and/or signalling. In our study, we discovered that both the H₄R(302) and H₄R(67) are non-functional, and expressed predominantly intracellular. However, when either of the H₄R splice variants was co-expressed with the full length H₄R(390) an apparent reduction in Bₘₐₓ could be observed. The reduction could be explained through the observation that co-expression of either splice variant retained the H₄R(390) intracellular, by forming hetero-oligomers. However, the hetero-oligomers were also present at the cell surface as determined by tr-FRET. It is possible that the H₄R-H₄R splice variant hetero-oligomers create some sort of H₄R receptor reserve; keeping the H₄R(390) inactive when little H₄R activity is required.

Oligomerization of aminergic GPCRs

Oligomerization occurs for receptors within each of the three major families of GPCRs. However, only for members of family C has oligomerization shown to be vital for receptor function. While in the last two decades over 300 articles have dealt with GPCR hetero-oligomerization, still many questions remain unanswered or have only been partially answered. For example, is there a general motif which is responsible for the GPCRs to form oligomers? At which stage do the GPCRs within the oligomers dissociate from each other and what is the percentage of receptors that are present within oligomeric complexes? Within the family A of rhodospin-like GPCRs, the oligomerization of the aminergic GPCRs has been extensively studied. Studies on the α- and β-adrenergic receptors have revealed that TMI and TMVI are of importance in the interaction between the receptors within the oligomeric complex. Studies on the dopamine D₂ receptors also indicated a role for TMIV. The discovery that the truncated H₄R(67), consisting only of TMI and part of TM II, was still able to hetero-oligomerize with the full length H₄R, emphasized the importance of this region in the oligomerization interface (chapter 4). Whereas the TM domains are involved in the direct interaction within in the oligomerization interface, the oligomeric complex may be stabilized via the glycosylated N-terminus. We indeed found evidence for a decrease in the amount
H₄R oligomers after deglycosylation of the receptors (chapter 3). This stabilizing role of N-glycosylation has been reported also for the bradykinin receptors 212. However, this is not necessarily a general feature as for dopamine D₃ receptors no decrease in the amount of receptor dimers was observed after deglycosylation 331.

**Hetero-oligomerization between the H₄R and human chemokine receptors**

The H₄R is involved in mediating the release of several cytokines 118,161,184 and shows substantial overlap in cellular expression with the family of chemokine receptors 427. Chemokine receptors are well known for their role in inflammation 192,193 and several cases of chemokine receptor oligomerization have been reported 269,368-372. To investigate if the H₄R is able to form hetero-oligomers with chemokine receptors, we screened all CC chemokine receptors, with the exception of CCR7 and CCR11, for their ability to hetero-oligomerize with the H₄R in a tr-FRET assay. Distinct tr-FRET signals were observed only for H₄R-CCR1, H₄R-CCR4 and H₄R-CCR8 hetero-oligomers (Figure 8.2). The physiological relevance of these apparent H₄R-CC chemokine receptor interactions is not yet clear. However, Buckland and co-workers already reported that histamine induced activation of the H₄R can potentiate the chemotactic effect of CCL11 167, CCL27 and CCL24 165. CCL11 (eotaxin) and CCL24 (eotaxin-2) are chemokines known to bind CCR3 427, whereas CCL7 (MCP-3) binds both CCR1 and CCR2 with high affinity 427. Therefore it is possible that the effect of potentiation of CCL7 chemotaxis may (partially) be attributed to H₄R-chemokine hetero-oligomerization. Further studies are required to obtain a better understanding of the physiological role of the H₄R-chemokine receptor interaction.
Liver expressed chemokine (CCL16), a ligand for H₄R?

In 2004 Nakayama and co-workers reported on a liver expressed chemokine (LEC, CCL16), which was able to bind with high affinity to the H₄R and able to induce Ca²⁺-mobilization and chemotaxis in murine L1.2 cells expressing the H₄R. In our attempts to reproduce these results of Nakayama and co-workers, we were unable to show displacement of \[^{125}\text{I}]\text{CCL16}\) using high dosage (10 μM) of unlabeled histamine both in COS-7 and L1.2 cells expressing recombinant H₄R (Figure 8.3A). It should be noted that...
Nakayama and co-workers used the Bolton-Hunter method\textsuperscript{166} to label CCL16, whereas we employed the iodogen method. The methods differ in the amino-acid to which the \(^{125}\text{I}\) is attached. However, we were also unable to show displacement of \(^{[3\text{H}]}\text{histamine}\) by CCL16 (Figure 8.3B). Moreover at concentrations of CCL16, which showed clear effects in both Ca\(^{2+}\)-mobilization as well as chemotaxis assays \textsuperscript{166} we were unable to induce a strong inhibition in CRE-driven luciferase production (Figure 8.3C). It is possible that CCL16 does not target the H\(_4\)R directly but that it binds for example the potential H\(_4\)R-CCR1 or H\(_4\)R-CCR8 hetero-oligomer. Further experiments will need to be performed to investigate this hypothesis.

\textbf{Figure 8.3:} Lack of effect of \(^{[125}\text{I}]\text{CCL16}\) on H\(_4\)R. A, \(^{[125}\text{I}]\text{CCL16}\) displacement by 10 \(\mu\text{M}\) histamine on COS-7 cells transiently expressing H\(_4\)R or murine L1.2 cells stably expressing H\(_4\)R. B, No displacement of \(^{[3\text{H}]}\text{histamine}\) by CCL16. C, Marginal effect of CCL16 on 1 \(\mu\text{M}\) forskolin induced inhibition of cAMP, measured using a CRE-luciferase reporter gene assay in COS-7 cells transiently expressing H\(_4\)R and a CRE-luciferase reporter gene. * = \(P<0.05\), **** = \(P<0.0001\). (HA, histamine; TP, thioperamide)

\textbf{Hetero-oligomerization between the H\(_4\)R and US28}

Several viruses carry genes encoding GPCRs, sharing high homology with chemokine receptors, within their genome\textsuperscript{373}. The genome of the human cytomegalovirus encodes four viral chemokine receptors, US28, US27, UL33 and UL78\textsuperscript{377}. Out of these four, US28 has been the most studied GPCR. So far, US28 has been shown to induce migration of smooth muscle cells, potentially linking it to atherosclerosis\textsuperscript{430}. Additionally, US28 may serve as co-receptor for HIV-1 entry\textsuperscript{431}. Moreover, recently US28 was identified as a potential oncogen, inducing a proangiogenic and transformed phenotype by up-regulating the expression of...
vascular endothelial growth factor and enhancing cell growth and cell cycle progression. US28 shares highest homology with CX3CR1. US28 may act as a chemokine scavenger, potentially preventing chemotaxis of leukocytes. In addition, co-expression of US28 with the G_{i/o}-coupled CCR1 chemokine receptor, potentiated a marked increase in G_{i/o}-sensitive CCL5 activation of NF-κB signalling. Moreover, US28 is also transcribed in latently infected monocytes. The G_{i/o}-coupled H_{4}R has been shown to induce chemotaxis of eosinophils and is also expressed in monocytes. Therefore it was of interest to investigate the interaction between the H_{4}R and US28. Using biophysical and biochemical approaches we determined that the hetero-oligomerization occurs between the H_{4}R and the US28. A reduction in H_{4}R binding was observed when co-expressed with the US28. Moreover, the co-expression increased US28 mediated NF-κB-signalling. The transcription factor NF-κB promotes expression of >100 genes, predominantly involved in the immune response. It appears that US28 tries to escape the immune system, by scavenging chemokines as well as altering immune related gene transcription and by blocking activation of chemotactic receptors through hetero-oligomerization. To further support this hypothesis, chemotaxis assays should be performed. In order to study this in a physiological setting, these experiments would preferentially be carried out in HCMV infected cells endogenously expressing the H_{4}R.

Many chemokine receptors signal via the JAK-STAT pathway. Activation of the H_{4}R may also cause modulation of the cytokine induced JAK/STAT. So far, no information is available whether US28 can activate JAK-STAT. Therefore, it will be interesting to study US28 mediated JAK-STAT signalling and the effect of hetero-oligomerization between the H_{4}R and US28 on this particular pathway.

Overexpression of the H_{4}R for structural studies
Currently, we are successful in producing large amounts of H_{4}R protein. Employing baculovirus expression system, heterologous expression in mammalian cells, as well as cell free expression using E.coli extracts, milligram amount of purified H_{4}R could be obtained. This purified H_{4}R will be used to study the structure of the receptor and binding pocket in more detail using spectrometric techniques such as
NMR (nuclear magnetic resonance), MS (mass spectrometry) and FT-IR (fourier-transformed infrared). In a more distant past we were also successful in overexpressing the H₂R using the Semliki forest virus.

The H₄R receptor is a potential drug target to treat diseases like rheumatoid arthritis, inflammatory bowel disease and allergic rhinitis. Therefore, to assist in the development of H₄R lead compounds specific structural information of the H₄R would be useful. In order to attain such information using spectrometric techniques, large amounts of pure H₄R protein need to be produced. Several methods to overexpress the H₄R have been evaluated and described in chapter 7. Each of the methods (baculovirus, Semliki Forest virus, codon optimization and epitope tagging) by itself did not result in expression levels (maximally 14 pmol/mg protein) that were comparable to the B_max obtained for the H₁R (>50 pmol/mg protein). However, it is plausible that combination of some of the methods will be able to increase the H₄R expression level. One of the options that could be investigated is to construct an N-terminally hemaglutinin (HA) codon optimized H₄R under a polyhedron promoter to use in the baculovirus expression system. Another option is the use of cell free expression, which has been successfully employed to produce mg amounts of the human β₂-adrenergic receptor and the hH₁R.

The use of the H₄coR, as described in chapter 7, would not be advised in this strategy as the H₄R was codon optimized for human, not for E.coli.

Concluding remarks

Since the original six papers, describing the cloning of the H₄R published in 2000/2001, the scientific output regarding the H₄R has increased almost ten fold in six year time, to over 50 articles. In these six years the involvement of the H₄R in inflammation, initially suggested based on its expression in hematopoietic cells, has been established. Potential involvements in other diseases such as colon cancer, breast cancer and rheumatoid arthritis have been put forward, making the H₄R an interesting potential drug target. Several selective agonists and antagonists have been identified, including 4-methylhistamine and VUF 6002 described within this thesis. Yet, besides these compounds the only other true selective compound is JNJ 7777120. H₄R ligands with subnanomolar
affinities and/or potencies, as are available for the H3R, are not (yet) available. The search for these ligands will benefit from more structural information on the H4R. In general this can be obtained using spectroscopical analysis techniques such as MS, NMR or FT-IR. However, this requires a relatively large and pure amount of H4R protein. We have shown that it is possible to obtain reasonable expression levels of the H4R in recombinant systems, to continue with further purification and analysis steps. Current work on the H1R shows that MS analysis is able to provide information on the receptors ligand binding pocket\textsuperscript{437}.

So far two and perhaps three (putative H4cR) H4R splice variants have been identified. Considering that the genomic arrangement of the H4R gene is similar to that of the H3R for which already twenty splice variants have been discovered\textsuperscript{124,130}, several more H4R isoforms may exist. The currently known H4R splice variants are able to interact and regulate functional H4R expression through hetero-oligomerization. However, other roles for these H4R isoforms may exist. The viral chemokine receptor US28 appears to interact with the H4R through hetero-oligomerization to potentially use (abuse) this receptor for its own benefit e.g. increased signalling and perhaps evading host defence mechanisms. It is possible that the US28 receptor mimics native H4R hetero-oligomeric partners. The H4R may form hetero-oligomers with other chemokine receptors including CCR1, which shares high homology with the US28 receptor. The interaction between the H4R and the chemokine system has been subject of several studies\textsuperscript{163,165,167,184}. However, many more studies are required to try and truly understand the physiological role and actions of the H4R herein.

All in all, much progress has been made in the understanding of the molecular pharmacology of the H4R, but still many questions remain unanswered, while new findings, often add more questions. Therefore, the H4R deserves to receive the same (if not more) attention and continuous research efforts it has received so far, for many more years to go.
De moleculaire farmacologie van de histamine H₄ receptor

De aandacht in dit proefschrift is gericht om een beter inzicht te krijgen in hoe de histamine H₄ receptor functioneert. De focus ligt hierbij op moleculair niveau. De histamine H₄ receptor is een G-eiwit gekoppelde receptor (GPCR). Dit type receptor speelt een belangrijke rol in ons lichaam, en vormen de bindingsplaats voor verschillende stimuli zoals licht, geur, endorfine, adrenaline en histamine. Naar schatting 50% van alle medicijnen op de markt binden aan deze GPCRs. De GPCRs zijn onder te verdelen in kleinere subpopulaties/families. Een subpopulatie van GPCRs vormen de histamine receptoren. De histamine receptor familie bestaat uit 4 leden. De histamine H₁ receptor is het langst bekend en speelt een rol in allergie. De histamine H₂ receptor is onder meer belangrijk in de secretie van maagzuur. De histamine H₃ receptor komt voornamelijk voor in de hersenen en is mogelijk betrokken bij verscheidene processen, zoals, voedsel inname, slaap/waak cyclus. De histamine H₄ receptor is het jongste lid van de histamine receptor familie en het DNA dat codeert voor het eiwit dat de H₄ receptor opmaakt is rond het jaar 2000 ontdekt en gekloneerd.

Tijdens de start van dit promotie onderzoek was er slechts weinig bekend over de rol van de histamine H₄ receptor. Om een idee te krijgen wat voor stoffen/liganden goed kunnen binden aan deze receptor zijn verschillende liganden, waarvan bekend is dat ze een interactie vertonen met tenminste een van de drie andere histamine receptoren, getest op de sterkte van hun affiniteit voor de histamine H₄ receptor. De ontdekking dat 4-methylhistamine, een ligand met middelmatige affiniteit voor de histamine H₂ receptor, een goede affiniteit heeft voor de H₄R is beschreven in hoofdstuk 2.

Verscheidene eiwitten in de natuur hebben de neiging om aan elkaar te plakken. Dit fenomeen staat bekend als oligomerizatie. Wanneer de eiwitten identiek zijn spreekt men over homo-oligomerizatie en wanneer de eiwitten van elkaar verschillen wordt dit aangeduid als hetero-oligomerizatie. Hoewel oligomerizatie
Samenvatting

van eiwitten al langer bekend was, werd over het algemeen aangenomen dat GPCRs als enkele units functioneerden. Pas sinds het eind van de 20ste eeuw is er sterk bewijs dat oligomerisatie echter ook voorkomt bij GPCRs. Hoofdstuk 3 beschrijft hoe met behulp van verschillende technieken is bepaald dat de H4R hoogstwaarschijnlijk ook kan oligomerizeren.

Eiwitten worden gecodeerd door genen in ons lichaam. Elk gen is een stuk DNA met een specifiek begin en einde. Voor sommige eiwitten, waaronder de H3R en H4R is het gen dat codeert voor het receptor eiwit niet een onafgebroken stuk DNA, maar is het verspreid in stukjes over een grotere afstand. Normaal gesproken verwijdert ons lichaam het DNA dat niet codeert voor het receptor eiwit en plakt alle stukken van het gen vervolgens aan elkaar. Echter, soms maakt het lichaam een fout en verwijdert het te veel of te weinig DNA, wat vervolgens een gen oplevert dat een iets anders receptor eiwit oplevert dan “normaal”. Het is bekend dat dit voor de H3R gebeurt. Hierdoor bestaan er in ieder geval 20 vormen van de H3R, die of langer of korter zijn dan de “normale” H3R.

Hoofdstuk 4 beschrijft de identificatie van kleinere vormen van de H4R. Deze kleinere H4Rs kunnen hetero-oligomers vormen met de “normale” H4R en hebben zodanig een negatieve invloed op het functioneren van de “normale” H4R.

De histamine receptoren behoren tot de speciale klasse van aminerge GPCRs. De GPCRs in deze klasse binden kleine liganden, die van aminozuren zijn afgeleid. In hoofdstuk 5 wordt een overzicht gegeven van alle gepubliceerde data, die de oligomerisatie van deze aminerge GPCRs betreft.

De chemokine receptoren zijn een klasse van GPCRs die een grote rol spelen in inflammatie. Recent onderzoek heeft uitgewezen dat de histamine H4 receptor een rol speelt in inflammatie. Bepaalde herpesvirusen zoals karposi’s sarcoma virus en humaan cytomegalovirus bevatten genen, die coderen voor chemokine receptoren. Een veel bestudeerde virale chemokine receptor is US28. Hoofdstuk 6 beschrijft hoe de H4R en US28 receptor hetero-oligomeren kunnen vormen en hoe dit de signaal transductie beïnvloedt.
Voor de ontwikkeling van nieuwe geneesmiddelen is het erg informatief om in detail de structuur van de GPCR te kennen. Om gedetailleerde informatie te krijgen is het noodzakelijk om relatief grote en zuivere hoeveelheden van het receptor eiwit te verkrijgen. Hoofdstuk 7 beschrijft hoe met onder andere gemodificeerde virussen geprobeerd is grote hoeveelheden H₄R the produceren.

Tenslotte word in hoofdstuk 8 getracht de bevindingen gedaan in dit promotie onderzoek in een breder perspectief te plaatsen. Verder worden suggesties gegeven voor mogelijke experimenten om het onderzoek naar de H₄R voor te zetten.

Hoewel er sinds de ontdekking van de H₄R vooruitgang is geboekt in het begrip van de rol van de H₄R blijven er voldoende vragen nog onbeantwoord. Tot dusver is nog niet bekend of de H₄R een cruciale rol speelt in een bepaald ziektebeeld, hoewel suggesties voor een rol in artritis en borstkanker zijn onlangs zijn gemaakt. Als de H₄R een belangrijk doelwit blijkt te zijn, zal dit de ontwikkeling van nieuwe liganden met hoge affiniteit voor de H₄R zeker doen bespoedigen. Met het oog op hetero-oligomerizatie is het misschien noodzakelijk of therapeutisch effectiever om liganden te ontwikkelen, die selectief binden aan het hetero-oligomeer.
References


194


60. Pan, L., Gurevich, E. V. & Gurevich, V. V. The nature of the arrestin-receptor complex determines the ultimate fate of the internalized receptor. J Biol Chem (2003).


106. Fukushima, Y. et al. Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. *Biochim Biophys Acta*** **1539**, 181-91 (2001).


186. Bell, J. K., McQueen, D. S. & Rees, J. L. Involvement of histamine H4 and H1 receptors in scratching induced by histamine receptor agonists in BalbC mice. Br J Pharmacol 142, 374-80 (2004).


References


References


295. Gines, S. et al. Dopamine D1 and adenosine A1 receptors form functionally


340. Novi, F., Scarselli, M., Corsini, G. U. & Maggio, R. The paired activation of the two components of the muscarinic M3 receptor dimer is required for induction of ERK1/2
References


References


416. Lundstrom, K. et al. High-level expression of the human neurokinin-1 receptor in mammalian cell lines using the Semliki


<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;-3R</td>
<td>adenosine A&lt;sub&gt;1&lt;/sub&gt;-3 receptor</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>BEVS</td>
<td>Baculovirus expression vector system</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine</td>
</tr>
<tr>
<td>COS-7</td>
<td>African green monkey cells</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovarian cell</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
</tr>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;-5R</td>
<td>dopamine D&lt;sub&gt;1&lt;/sub&gt;-5 receptor</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DOR</td>
<td>δ opioid receptor</td>
</tr>
<tr>
<td>Dpi</td>
<td>days post infection</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EL</td>
<td>extracellular loop</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>eYFP</td>
<td>enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier-transformed infrared spectroscopy</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>g protein-coupled receptor</td>
</tr>
<tr>
<td>H&lt;sub&gt;1&lt;/sub&gt;-4R</td>
<td>histamine H&lt;sub&gt;1&lt;/sub&gt;-4 receptor</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEK 293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HEK 293T</td>
<td>T-antigen activated HEK 293 cells</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>Insp3</td>
<td>inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>JNJ7777120</td>
<td>1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine</td>
</tr>
<tr>
<td>KOR</td>
<td>κ opioid receptor</td>
</tr>
<tr>
<td>M&lt;sub&gt;1&lt;/sub&gt;-5R</td>
<td>muscarinic M&lt;sub&gt;1&lt;/sub&gt;-5 receptor</td>
</tr>
<tr>
<td>MT&lt;sub&gt;1&lt;/sub&gt;-2R</td>
<td>melatonin MT1-2 receptor</td>
</tr>
<tr>
<td>MOR</td>
<td>μ opioid receptor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance spectroscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RLuc</td>
<td>Renilla luciferase</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugiperda 9 cells</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>tr-FRET</td>
<td>time-resolved Fluorescence Resonance Energy Transfer</td>
</tr>
</tbody>
</table>
Acknowledgements

While it is almost customary to thank ones PhD supervisor first, I would like to take this opportunity to thank people who influenced my decision to start work as a PhD. I would like to thank Prof. Dr. Rob Verpoorte for allowing me to work for a year as undergraduate in his lab, which really initiated my feeling for performing science in a university environment. Secondly, I would like to thank Sjoerd van den Worm for showing me what it can be like to work as a PhD student. As an undergraduate in the group of Prof. dr. Cees Pleij I witnessed how three PhD’s students (together with Sharif Barends and Hugo Bink) can combine their knowledge and enthusiasm, which eventually resulted in the publication of an article in the very prestigious journal “Cell”.

I would like to thank Prof. Dr. Rob Leurs for giving me the chance to perform a PhD project of my own within his lab on the molecular pharmacology of the then recently discovered histamine H₄ receptor. Also Rob, you showed me the importance in keeping your focus on the path to publication when necessary, as well as how to write down your data in a way that will capture the attention of the scientific community.

Remko, I am thankful for making me feel comfortable to disturb you at any time of the day if I had a question about my project, interpretation of data. You always managed to be a step ahead in finding interesting articles. Often I couldn’t keep up reading all the articles you forwarded me.

Paul I am very grateful for the opportunity you gave me to perform important immunoblotting experiments in your lab. The data presented in this thesis would not nearly be as convincing if it wasn’t for all your collaborative efforts. And it is an honour for me to have you being a member of the thesis defense. In the same breath I would like to thank Fiona, for her hospitality and kindness, as well as helping me with the immobilization assay, and performing several experiments, which ended up in this thesis.

I would like to thank Wim, Jenny and Petra for giving me the opportunity to learn how to work with baculovirus in Nijmegen.
I would like to thank Andre for sharing his knowledge and troubleshooting skills in the field of molecular biology, which have been very useful, I still make sure I always have a copy of the New England Bioscience “catalogue/bible” close at hand. Also thanks for getting me a job at Biofocus (I leave it up to you whether or not I say this in an ironic way 😁).

Thanks Herman for being my H4 partner in crime, even if our working hours did not always overlap. I has been very nice being able to discuss any idea about H4 to you. I am very happy you agreed to be one of my paranimfen.

A big round of applause for all the people in the pharmacochemistry department: Aloys, Martijn, Dennis, Carlos, Silvina, Franka, Saskia, Obbe, Paola, Martine, Henry, Ruengwit

Nalan, Andrea, Janneke, Rogier, Antonio, Ellen, David, Marola as well as Kees, Qin and Elisabeth and anybody I may have forgotten to mention.

I like to thank the students I have supervised and have in one way or another contributed to the work performed in this thesis: Petra de Kruijf (clozapine analogues), Kristina Simonyan (dimeric ligands), Barbera Stam (overexpression), Mirabella Ponczi (codon optimized H4), Angelique Ramlal (H4-US28 hetero-oligomerization) and Jan Simon Boerma (H4-chemokine receptor oligomerization).

I would like to thank Jennifer Whistler for allowing me to start as a post-doc in her lab even though I still hadn’t completed writing my thesis yet.

Jib, to you I am most grateful, you above anyone else gave me the strength and motivation to work as a PhD student. No matter what happened I could rely having you on my side. Maybe I should regret having you start your own PhD project within the VU, but it was really nice having you work so close to me. Often I found you sitting behind my computer and my lunchbox missing several pieces of bread. Life of a PhD has its highs and lows; I know we both saw our share of it, still in the end I tend to remember only the good times. I hope from the bottom of my heart you will finish your own PhD project on a high.
Curriculum vitae

Richard Michiel van Rijn was born June 8 1979 in Abbenes (The Netherlands). He attended the “Voorbereidend Wetenschappelijk Onderwijs” at the Herbert Vissers College in Nieuw-Vennep. After his graduation he studied bio-pharmaceutical sciences at Leiden University in 1997. During the final stage of his study he performed a one year internship within the department pharmacognosy (Leiden/Amsterdam Center for Drug Research) in the group of prof dr. R Verpoorte, isolating acetylcholine esterase inhibitors from amaryllidaceae bulbs, under direct supervision of dr. I.K. Rhee. He performed an additional six month internship within genexpress (Leiden Insitute of Chemistry), led by prof. dr. C.W.A. Pleij. The work focused on the bacteriophage AP205 and was supervised by dr. S.H.E. van der Worm.

After graduation in 2002, he immediately continued his study with his Ph.D. project in the lab of prof. dr. R. Leurs (pharmacochemistry, LACDR). The performed work, described within this thesis, focused on the recently discovered histamine H_4 receptor, with the emphasis on the molecular pharmacological aspects of the receptor. The work was supervised by dr. R. Bakker. Within the Ph.D. project he collaborated with and visited the labs of prof. dr. W. de Grip (Radboud University Nijmegen, The Netherlands) and dr. P. L. Chazot (Durham University, United Kingdom). From June till December 2006 he was employed as associate scientist at Biofocus DPI, a Galapagos company focused on target discovery trough the use of adenoviral knockdown libraries. Since January 15 2007 he is working at the Ernest Gallo Clinic and Research Center of the University of San Francisco (UCSF) as post-doctoral researcher within the lab of dr. Jennifer Whistler.
List of publications


Van Rijn RM1, Rhee IK1, Verpoorte R., Isolation of acetylcholinesterase inhibitory alkaloids from Nerine bowdenii, Manuscript submitted to Fitoterapia