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Roland Seifert *Editors*

Histamine and Histamine Receptors in Health and Disease

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Preface

The last volume on histamine in the *Handbook of Experimental Pharmacology*, entitled *Histamine and Histamine Antagonists*, was published as Volume 97 in 1991 and edited by Börje Uvnäs. This seminal volume summarized the state of the art in histamine research with a focus on methods for histamine determination, regulation of histamine release from mast cells, histamine metabolism, histamine receptors in the brain, histamine in the cardiovascular and gastrointestinal system, the development of histamine H_1-receptor antagonists of the second generation as well as the development of histamine H_2-receptor antagonists for the treatment of gastroduodenal ulcer disease.

Since then, more than 25 years passed, and the Editors of this volume felt that again, it is time to review the field of histamine research. This is easier said than done because histamine research is very complex and encompasses researchers from very different directions and philosophies, reflecting the fact that histamine plays a role in so many (patho)physiological processes. Thus, the Editors are fully aware of the fact that this book cannot provide a complete overview of the entire field of histamine research. Rather, the book tries to highlight selected aspects of the field by leading experts, some more junior and some more senior, in the respective fields in a balanced manner. We tried to integrate scientists from various continents with distinct cultural approaches to the field. Every author was asked to put her/his research into a broader perspective and outline future directions of research.

This book gives an overview of new sensitive methods for histamine detection and emphasize major achievements on the molecular characterization of histamine receptors as well as histamine and histamine receptors in disease contexts. Since the last volume on histamine in this series, four histamine receptors have been cloned and characterized with methods from the fields of molecular pharmacology, molecular biology, and medicinal chemistry. Mouse gene knockout models have tremendously enhanced our knowledge on the function of the four histamine receptor subtypes. A highlight in the field has been the recent crystallization of the histamine H_1-receptor. Important roles of histamine and histamine receptors in diseases, including allergies, food intoxication, acute myelogenous leukemia, Tourette syndrome, and narcolepsy, are discussed in this book. The first histamine H_3-receptor antagonist has been approved for clinical use, even histamine has become a drug, and histamine H_4-receptor antagonists are in the clinical development. All these exciting aspects are covered in this book.

We really appreciate the commitment of the authors to write and revise their contributions in due time.

We do hope that this book will guide the large and diverse international community of histamine researchers in conducting productive and critical research in the field.

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Analytical Methods for the Quantification of Histamine and Histamine Metabolites

Heike Bähre and Volkhard Kaever

Contents

Abstract

The endogenous metabolite histamine (HA) is synthesized in various mammalian cells but can also be ingested from exogenous sources. It is involved in a plethora of physiological and pathophysiological processes. So far, four different HA receptors (H_1R–H_4R) have been described and numerous HAR antagonists have been developed. Contemporary investigations regarding the various roles of HA and its main metabolites have been hampered by the lack of highly specific

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and sensitive analytic methods for all of these analytes. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the method of choice for identification and sensitive quantification of many low-molecular weight endogenous metabolites. In this chapter, different methodological aspects of HA quantification as well as recommendations for LC-MS/MS methods suitable for analysis of HA and its main metabolites are summarized.

Keywords
Histamine • Histamine metabolites • HPLC • Mass spectrometry

1 Introduction

Histamine (HA), chemical name 2-(4-imidazolyl)-ethylamine, represents an important mediator of many biological processes. Due to its potent activity already at low concentrations, its synthesis, storage, release, and metabolism have to be strictly controlled in order to avoid unwanted reactions.

HA occurs to various degrees in foods, e.g., in tuna (Self et al. 2011) and increases with maturation in the presence of histidine decarboxylase-positive microorganisms. Therefore, high concentrations of HA are found in many fermented foodstuffs and beverages, such as aged cheese and red wine (Garcia-Villar et al. 2009; Cunha et al. 2011), in significant amounts. This can lead to food poisoning (Colombo et al. 2016) and HA intolerance (Maintz und Novak 2007). Especially persons with low extracellular amine oxidase expression or patients taking amine oxidase-inhibiting drugs are at risk of HA toxic effects mimicking the symptoms of type I allergic reactions.

Physiological effects of HA were already described more than 100 years ago (Dale and Laidlaw 1910). They demonstrated that HA causes vasodilatation, contraction of smooth muscles in the airways, uterus, and the intestine, stimulates heart rate and contractility, and induces a shock-like syndrome when injected into animals. Later on, the effect of HA on hydrochloric acid secretion of the stomach and its additional roles in neurotransmission, immunomodulation, hematopoiesis, wound healing, day–night rhythm, and the regulation of HA-induced cell proliferation and angiogenesis in tumor models and intestinal ischemia were described (Maintz and Novak 2007; He et al. 2012; Cataldi et al. 2014).

HA exerts its effects via different G protein-coupled HA receptors (H_1R, H_2R, H_3R, and H_4R) and, therefore, specific HAR agonists and antagonists have been developed (Thurmond et al. 2008; Cataldi et al. 2014).

The availability of reliable detection methods for HA and its main metabolites is an absolute requirement for further research in this field. Ideally, simultaneous quantification of several metabolites should be feasible. Numerous detection methods for HA and its main metabolites have already been described, i.e., immunological assays, such as radioimmunoassay or ELISA (Guesdon et al. 1986; McBride et al. 1988; Gill et al. 1991; Poli et al. 2016) and chromatographic methods (Oguri and Yoneya 2002; Toyo'oka 2008; Wang et al. 2013) including capillary

electrophoresis (Nishiwaki et al. 2000; Simo et al. 2008; An et al. 2016), gas chromatography (Martens-Lobenhoffer and Neumann 1999; Pittertschatscher et al. 2002; Cunha et al. 2011; Husek et al. 2016), LC with fluorescence (Miyamato et al. 2004; Hogan et al. 2012; Wang et al. 2013), LC with electrochemical detection (Jensen and Marley 1995; Maldonado and Maeyama 2012), and LC-MS/MS (Garcia-Villar et al. 2009; Koyama et al. 2009; Croyal et al. 2011; Self et al. 2011; Zhang et al. 2011; Zimmermann et al. 2011; Wang et al. 2013; Liu et al. 2014; Chimalakonda et al. 2015; Tschirner et al. 2015; Laurichesse et al. 2016; Poli et al. 2016; Tschirner et al. 2016) (see Sect. 3). However, adequate sample preparation steps resulting in an instant stop of cellular metabolism and removal of interfering matrix components are of comparable significance (Vuckovic 2012).

2 Histamine Metabolism

HA is generated by decarboxylation of the amino acid L-histidine. This enzymatic reaction is catalyzed by the cytosolic histidine decarboxylase (HDC, EC 4.1.1.22), an enzyme that is only detectable in cells that actively produce HA. After HA synthesis, these cells (e.g., mast cells, basophils, enterochromaffin-like cells in the gastric mucosa, and histaminergic neurons) store HA in special intracellular granula (Oguri and Yoneya 2002). However, numerous other cell types such as lymphocytes or epithelial cells also synthesize small amounts of HA, which in these cells is not stored but, in contrast, immediately released.

The main steps of HA metabolism in mammals are depicted in Fig. 1 (Kyoto Encyclopedia of Genes and Genomes, KEEG, http://www.kegg.jp, downloaded on February 9th, 2017). As a major route of HA metabolism in the central nervous system, and to a lower extent in peripheral organs, the enzyme histamine-*N*-methyl transferase (HMT, EC 2.1.1.8) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the secondary amino group of HA. 1-Methylhistamine (1-MH), also named *N-tele*-histamine, is formed as product. This *N*-methylation of HA obviously involves the main step of HA inactivation, as the affinity of 1-MH to HA receptors is considerably lowered compared to that of HA. Within a next step, 1-MH is converted by a monoamine oxidase type B (MAO B, EC 1.4.3.4), leading to 1-methylimidazole-4-acetaldehyde (1-MI4A), which is further metabolized to 1-methylimidazole-4-acetic acid (1-MI4AA) by a NADP-dependent aldehyde dehydrogenase (EC 1.2.1.3).

Especially in peripheral organs HA can be directly metabolized by oxidative deamination (diamine oxidase, EC 1.4.3.22) of the primary amino group (Maslinski and Fogel 1991). The formed metabolite imidazole-4-acetaldehyde is rapidly oxidized by a NAD-dependent aldehyde dehydrogenase (EC 1.2.1.3), resulting in the formation of imidazole-4-acetic acid (I4AA). I4AA still contains some biological activity, e.g., as $GABA_A$ receptor agonist or $GABA_C$ receptor antagonist. In a last step, inactivation of I4AA is performed by ribosylation to 1-ribosylimidazole-4-acetic acid, catalyzed by the enzyme imidazoleacetate-phosphoribosyldiphosphate ligase (EC 6.3.4.8).

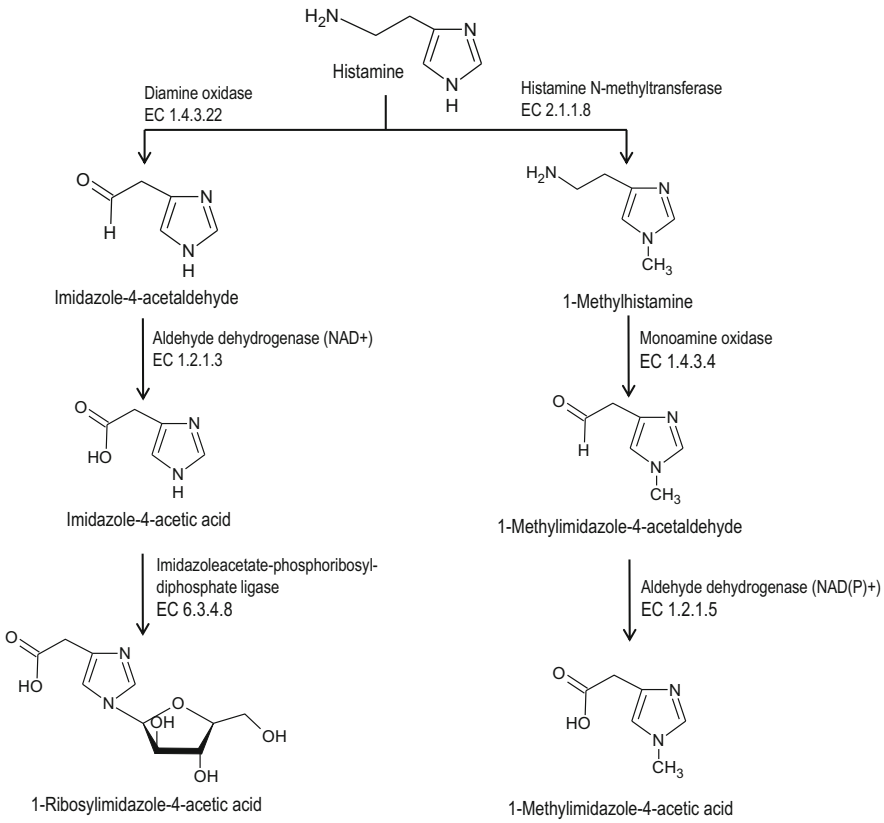

Fig. 1 Histamine metabolism (see detailed description in Sect. 2)

3 Analytical Methods for Histamine Quantification

In the following sentences different analytical methods for quantification of histamine and its main metabolites are described. Specific benefits and drawbacks of the specific methods are briefly summarized in Table 1.

3.1 Immunological Methods (RIA/ELISA)

Radioimmunoassay (RIA) has been considered as the gold standard method to quantify HA due to its high sensitivity of about 0.1 nM, provided that an acetylation step of HA is included (Gill et al. 1991). Plasma HA concentration of 0.3–2.0 nM was determined in healthy controls using this method. However, RIA is time-consuming and produces radioactive wastes (Poli et al. 2016). Alternatively, enzyme-linked immunoassays (ELISA) for HA have been described (Guesdon

Table 1 Benefits and drawbacks of different analytical methods for quantification of histamine and its metabolites

	Benefits	Drawbacks
Immunological methods	Gold standard	Time-consuming
RIA/ELISA	High sensitivity (for acetylated histamine)	Radioactive waste (RIA)
	Commercially available kits	Variable cross-reactivities
		Only one analyte per assay
Chromatographic methods	Numerous methods described in the literature	Prone to interferences by matrix components
	Simultaneous quantification of several analytes	
Capillary electrophoresis (CE)	High separation power	Insufficient sensitivity (without coupling to MS)
		Poor precision
Gas chromatography (GC)	Applicable to polar compounds	Derivatization of the analytes needed
Liquid chromatography (LC)	Different separation materials available (RP, HILIC)	LC method development challenging for polar compounds
Detection methods		Prone to interferences by matrix components
UV detection	Low detector costs	Very low sensitivity
Fluorescence detection	Sufficient sensitivity	Derivatization of the analyte needed
Electrochemical detection	Sufficient sensitivity	Poor reproducibility
Mass spectrometric detection	Highest sensitivity	High acquisition and maintenance costs
	Highest specificity	Highly experienced stuff needed
	Deuterated histamine available as internal standard	

et al. 1986) that are commercially available from different vendors and can simply be performed according to the manufacturer's instructions. All immunoassays have the disadvantage that only one specific analyte can be analyzed in the same assay. In addition, cross-reactivities with similar analytes, as, for example, with 1MH, have to be considered (Koyama et al. 2009). Because HA is a low-molecular weight molecule, and therefore a hapten with antigenic but without immunogenic potency, the production of specific anti-HA antibodies with high affinity is difficult (Wang et al. 2013).

3.2 Chromatographic Methods

Excellent summaries of analytical methods for HA and its metabolites including microseparation techniques, such as capillary electrophoresis (CE) and capillary electrochromatography (CEC), gas chromatography (GC), and liquid chromatography (LC), have previously been published (Oguri and Yoneya 2002; Toyo'oka 2008; Wang et al. 2013). These powerful techniques enable the separation of HA and its metabolites. Coupled to sensitive detection modes, e.g., fluorescence or mass spectrometry (MS), they offer simultaneous separation and highly sensitive detection. MS represents the most attractive detection mode for HA and its metabolites, at which different MS devices such as ion trap (IT), quadrupole (Q), and time-of-flight (TOF) systems have been applied (Toyo'oko 2008).

3.2.1 Capillary Electrophoresis and Capillary Electrochromatography

CE and CEC methods for HA have been described in the past, but they all suffer from insufficient sensitivity, due to the extremely low injection volume and the short pathlength of the irradiated light. The other disadvantage is the low precision of the migration time (Toyo'oka 2008). However, applying CE coupled to IT- or TOF-MS, HA could be quantified with high sensitivity in wine without any previous treatment steps except diluting with water and filtering (Simo et al. 2008). A lower limit of detection (LOD) value of 10 ng/mL was achieved, which is comparable to most LC fluorescence detection methods. Very recently, a newly developed electrochemiluminescence (ECL) sensing system for HA coupled to CE was reported (An et al. 2016). Under optimized conditions quantitative analysis of HA was achieved with an LOD of 1 ng/mL. However, it has to be proven in the future, whether this method will be applicable for HA quantification in complex matrices, such as plasma or tissues.

3.2.2 Gas Chromatography

When analytes are directly injected into a GC system, the main problem is adsorption, which results in peak tailing and a memory effect. To overcome both of these effects, suitable derivatization of the HA amine groups is employed, as, for example, using pentafluorobenzyl bromide for primary amino group derivatization (Oguri and Yomeya 2002). For derivatization of secondary amino groups, acetic anhydride and methylchloroformate were used to introduce an acetyl and a methoxycarbonyl group, respectively. Various detection devices have been employed for GC systems, including hydrogen flame ionization detector (FID), electron-capture detector (ECD), nitrogen–phosphorus detector (NPD), and MS as the best detection device with high selectivity and sensitivity. In this case, HA derivatives were ionized by electron impact (EI) or chemical ionization (CI) (Oguri and Yomeya 2002). As a good example for GC-MS analysis of HA in mouse plasma and culture supernatants of rat basophil leukemia cells, a validated one-step method including extractive derivatization of HA with ethylchloroformate in chloroform with an LOD of 2 ng/mL has been described (Pitterschatscher et al. 2002). Similar derivatization steps have been developed for the major HA

metabolites 1-MH and 1-MI4AA (Martens-Lobenhoffer and Neumann 1999), applying trifluoroacetic acid anhydride or pentafluorobenzyl bromide as derivatization reagents, respectively. Concentrations of about 2 and 20 μM were determined for 1-MH and 1-MI4AA in human urine. In a very recent manuscript an extractive derivatization method, combining derivatization and liquid–liquid microextraction prior to GC-MS analysis, was described for numerous urinary amino-carboxylic metabolites (Husek et al. 2016). An LOD of about 100 nM was specified for HA.

3.2.3 Liquid Chromatography

One of the advantages of LC compared to GC is that LC can also be applied to samples, which are unstable at high temperatures. In order to separate HA and its metabolites, reversed-phase (RP) material, often in combination with ion-pairing reagents, was applied (Oguri and Yoneya 2002). However, the concentrations of ion-pairing reagents have to be carefully chosen, as they often reduce analyte ionization, resulting in a decreased sensitivity of the assay. Alternatively, instead of RP chromatography hydrophilic interaction liquid chromatography (HILIC) can be used, although the HILIC material often shows diminished chemical stability compared to RP materials. A further increase in sensitivity can also be achieved using UPLC instead of classical LC systems.

Several detection devices such as UV absorption, fluorescence, electrochemical detection, and chemiluminescence have been applied in combination with LC. The main disadvantage of UV detection is the low sensitivity for HA analysis. Therefore, pre-, on-, and post-column derivatization steps have generally been included, e.g., with o-phthalaldehyde (OPA) for HA and 1-MH in murine brain (Miyamoto et al. 2004). Besides OPA, dansylchloride and additional reagents have been described for HA derivatization and subsequent fluorometric detection (Wang et al. 2013). The most sensitive UPLC-fluorescence method for quantitation of HA in human urine included derivatization of the primary and secondary amino moieties of HA with 4-(1-pyrene) butyric acid *N*-hydroxysuccinimide ester, leading to an LOD value of 0.04 nM (Hogan et al. 2012). Especially in the field of neurotransmitter and HA research, LC coupled with electrochemical detection (LC-ECD) has often been applied (Jensen and Marley 1995; Maldonado and Maeyama 2012). Although a high detection sensitivity was obtained from the ECD determination of standard compounds, the sensitivity in real sample analysis was often unconvincingly due to interferences by endogenous matrix components (Toyo'oka 2008). In the last years LC coupled to tandem mass spectrometers (QqQ) (Garcia-Villar et al. 2009; Koyama et al. 2009; Croyal et al. 2011; Zhang et al. 2011; Zimmermann et al. 2011; Wang et al. 2013; Liu et al. 2014; Chimalaconda et al. 2015; Tschirner et al. 2015, 2016) or high resolution mass spectrometers (HRMS) (Self et al. 2011; Poli et al. 2016; Laurichesse et al. 2016) have been applied in HA research. Irrespective of the used MS device, lower limits of quantification (LLOQ) ranging from 1 to 10 nM were obtained for HA. HRMS systems provide higher mass accuracy than QqQ mass spectrometers but, on the other hand, the linear calibrator concentration rage is diminished.

3.3 Mass Spectrometric Methods

Different MS devices coupled to GC (see Sect. 3.2.2) or LC (see Sect. 3.2.3) have been established for quantitation of HA and its metabolites. In LC analysis molecules are separated of each other due to their chemical structure and will reach the ionization source of the mass spectrometer. In HA analysis electrospray ionization (ESI) is commonly used in positive ionization mode, which leads to droplets containing positive charged ions (Garcia-Villar et al. 2009; Koyama et al. 2009; Croyal et al. 2011; Self et al. 2011; Zhang et al. 2011; Zimmermann et al. 2011; Wang et al. 2013; Liu et al. 2014; Chimalakonda et al. 2015; Tschirner et al. 2015; Laurichesse et al. 2016; Poli et al. 2016; Tschirner et al. 2016). These droplets shrink due to heat-induced desolvation until the repulsive force inside the droplets becomes too strong. The resulting Coulomb explosion finally leads to gaseous ionized molecules. In tandem mass spectrometric systems, the ions are accelerated towards the first quadrupole (Q1) of the mass spectrometer. Ions are separated according to their mass to charge ratio (m/z) and only those ions with a preset m/z-value are able to pass Q1 on a stable trajectory. All other ions will be discharged at the rods of the quadrupole. After passing the first quadrupole the so-called precursor ions enter the collision cell (q2) of the tandem mass spectrometer where a fragmentation takes place. In this reaction, the precursor ions collide with an inert collision gas (usually nitrogen or argon). The collision results in various fragment ions, which are now accelerated towards the third quadrupole (Q3). Only analyte-specific fragments are enabled to pass through to the detector. As a consequence, the resulting chromatogram only shows signals of selected analyte and their specific mass transitions (Fig. 2) (Table 2).

Despite this high selectivity, analysis may be influenced by coeluting matrix components in various ways (Taylor 2005). On the one hand, the mass transition of a matrix component may be so similar to, e.g., the HA mass transition that the resolution of the mass spectrometer is not high enough to discriminate between the matrix component and HA. Those unspecific matrix signals may falsify the result. To ensure correct peak identification the so-called quantifier/identifier ratio can be taken into consideration.

The fragmentation in the collision cell usually results in various fragments of one precursor ion with an analyte-specific fragmentation pattern. The fragment which shows highest intensity usually is used for quantification and is therefore called “quantifier.” To improve the reliability of an analysis, additional fragments (“identifier” or “qualifier”) can be detected to calculate the quantifier/identifier ratios. Only those signals showing the analyte-specific quantifier/identifier ratio should be used for quantification.

For HA, the main fragment (m/z 95.1) is generated by loss of the ammonia group. A second prominent fragment (m/z 67.8) is the result of a loss of the aminoethyl group (Koyama 2009). Since the ammonia loss shows higher intensity, it is usually used for quantification (Koyama 2009; Liu et al. 2014; Laurichesse et al. 2016). However, in biological samples the loss of this group is a quite unspecific mass transition. Therefore, the usage of the second fragment (m/z 67.8) might improve reliability of the HA analysis.

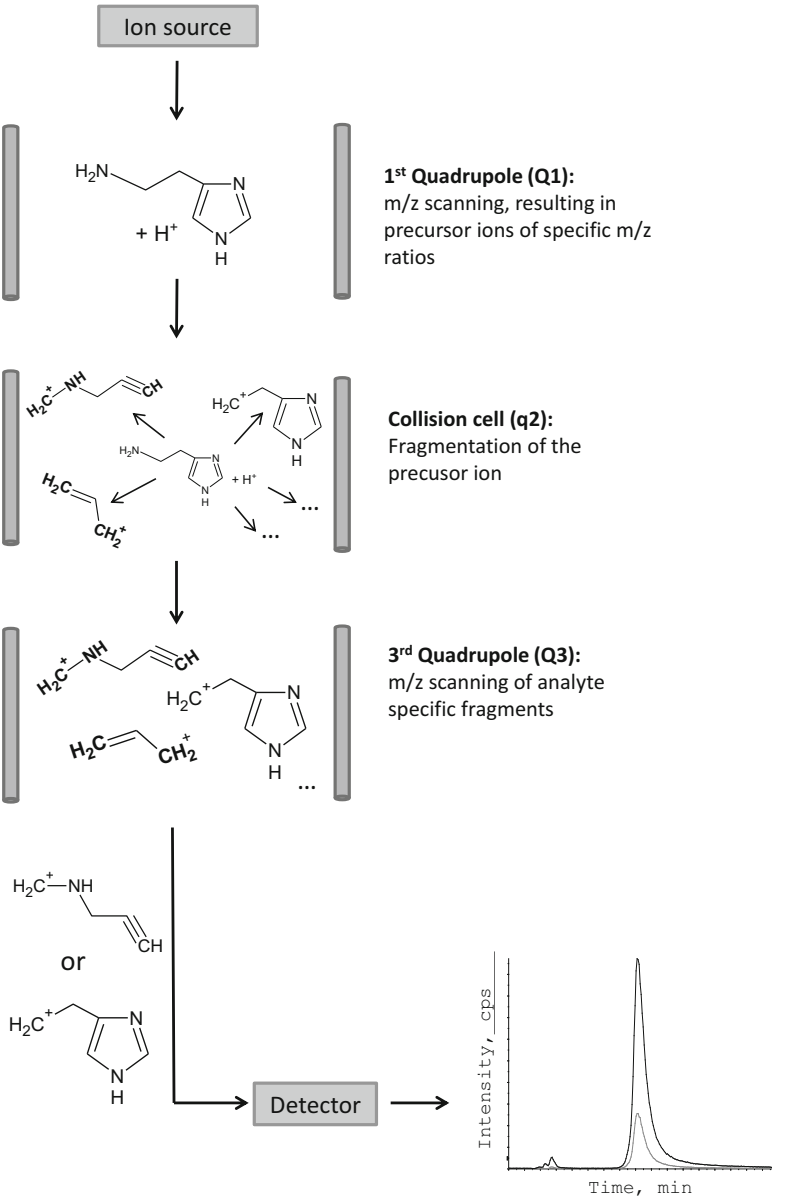

Fig. 2 Mass spectrometric analysis of histamine. After chromatographic separation and subsequent ionization, a mixture of molecules (including HA) enters the first quadrupole (Q1) of the mass spectrometer. Due to its specific mass-to-charge ratio ($m/z = 112.1$) HA is selected and enabled to pass through Q1. In the collision cell (q2), HA is dissociated to fragments by colliding with a collision gas (argon or nitrogen). Specific HA fragments (e.g., $m/z = 95.1$ and $m/z = 67.8$) can now be selected in the third quadrupole (Q3). They will reach the detector and finally give a specific signal in a chromatogram

Table 2 Mass spectrometric parameters applied for quantification of histamine, its main metabolites, and the internal standard d4-histamine

Analyte	*m/z* (precursor ion)	*m/z* (fragment ions)	Dwell time (ms)	DP (V)	CE (V)	CXP (V)
Histamine	112.1	95.1	80	30	23	12
		67.8	80	30	29	12
1-Methylhistamine	126.2	109.1	80	30	20	10
		67.1	80	30	40	10
Imidazole-4-acetic acid	127.1	**81.0**	80	30	20	10
		54.0	80	30	50	8
1-Methylimidazole-4-acetic acid	141.1	**95.1**	80	30	20	8
		68.1	80	30	40	8
d4-Histamine (IS)	116.1	**99.1**	80	30	23	11
		71.9	80	30	20	11

Quantifier transitions are marked in bold. *DP* declustering potential, *CE* collision energy, *CXP* collision cell exit potential

The described procedure is limited by the intensity of the less intense mass transition. Therefore, the signal of one mass transition might be too marginal for ratio calculation at low concentration ranges.

Matrix components may not only cause problems by additional signals in the chromatogram. A difference, e.g., in salt concentration between samples and calibrators can cause a shift in analyte retention time and, therefore, can complicate a reliable peak identification. Additionally, sample matrix may influence analyte ionization efficiency. In most cases the presence of matrix components during ionization leads to a decreased analyte ionization and, as a consequence, to reduced signal intensities resulting in a loss in sensitivity. If the matrix influence is different for calibrators and biological samples, respectively, a reliable analyte quantification is not possible. Therefore, the usage of an appropriate internal standard and calibrator matrix, which mimics the sample condition, is highly recommended.

The internal standard (IS) plays an essential role In LC-MS/MS analysis on the one hand to normalize variations during the sample preparation procedure, and on the other hand to minimize the matrix effects described above. Therefore, the chemical structures of the IS and the analyte should be as similar as possible. This ensures a comparable retention time as well as ionization efficiency of the IS and the analyte. By using peak area ratios of the analyte and the IS, and not only the peak area of the analyte, variations during sample preparation as well as variations in ionization can be compensated.

Stable isotopes-labeled IS show, due to the identical chemical structure, the same chromatographic behavior and ionization efficiency as its corresponding analyte. Therefore, a stable isotope-labeled IS reflects matrix effects best. Deuterated HA (d4-HA) is commonly used in HA analytics (Zimmermann et al. 2011; Liu et al. 2014; Chimalakonda et al. 2015; Tschirner et al. 2015, 2016; Laurichesse et al. 2016). Furthermore the usage of pyrazole (Koyama et al. 2009) and metformin (Self et al. 2011) as IS for HA LC-MS/MS analysis has been described.

Since HA and its degradation products do occur in most biological matrices, for most applications it is not possible to use exactly the same matrix for calibrators. In those cases the usage of an artificial or surrogate matrix (e.g., bovine serum albumin or artificial urine and cerebrospinal fluid) may help to compensate for the matrix effects described above (van de Merbel 2008; Zhang et al. 2011; Laurichesse et al. 2016). As an alternative approach, a surrogate analyte, in most cases a stable isotope-labeled standard, can be used (Jones et al. 2012; Liu et al. 2016). However, to our knowledge such an approach has not been described for HA so far.

4 Histamine Analyses by LC-Mass Spectrometry in Murine Samples

4.1 Mouse Tissues

Quantification of HA and 1-MH by LC-MS/MS has been described in organs from two common laboratory mouse strains with a lower limit of detection (LOD) of 0.2 pmol/mg organ weight (Zimmermann et al. 2011). HA was detectable in virtually all mouse organs, not only in those traditionally associated with HA-mediated disease.

In a follow-up study quantification of HA and its main metabolites 1-MH, 1-MI4AA, and 1-M4AA in brain extracts from HPRT-deficient mice and wild-type controls by LC-MS/MS analysis was recently described (Tschirner et al. 2015). In brief, different parts of the brain were homogenized using a FastPrep®-24 instrument [MP Biomedicals (Santa Ana, CA, USA)] after addition of an ice-cold extraction solvent, consisting of acetonitrile/water (4/1, v/v) (HPLC gradient grade, obtained from J.T. Baker, Deventer, The Netherlands), containing 0.2% (v/v) formic acid (Merck, Darmstadt, Germany) and 0.2 μM d4-HA (TLC PharmaChem, Ontario, Canada) as internal standard. Afterwards, samples were centrifuged and supernatant fluids were utilized for LC-MS/MS analysis. In this case, hydrophilic interaction liquid chromatography (HILIC) in combination with a QTRAP® 5500 tandem mass spectrometer (Sciex, Framingham, Massachusetts, USA) equipped with an electrospray ionization source operated in positive ionization mode was applied. An isocratic flow was used with a flow rate of 0.8 mL/min. The eluent was composed of 20% of 50 mM ammonium formate (Fluka/Sigma-Aldrich, St. Louis, Missouri, USA) in water supplemented with 0.2% (v/v) formic acid and 80% of acetonitrile with 0.2% (v/v) formic acid as well. A NUCLEODUR® HILIC column (125 mm × 2 mm, 3 μM particle size) (Macherey-Nagel, Düren, Germany) was used. The injection volume was 10 μL. Mass spectrometer settings were adjusted as described (Tschirner et al. 2015).

A representative chromatogram of a calibrator containing HA and its main metabolites 1-MH, 1-MI4AA, and I4AA is shown in Fig. 3. The final concentrations of HA and its metabolites were 0.7 μM in water. D4-HA (0.2 μM) was included as internal standard.

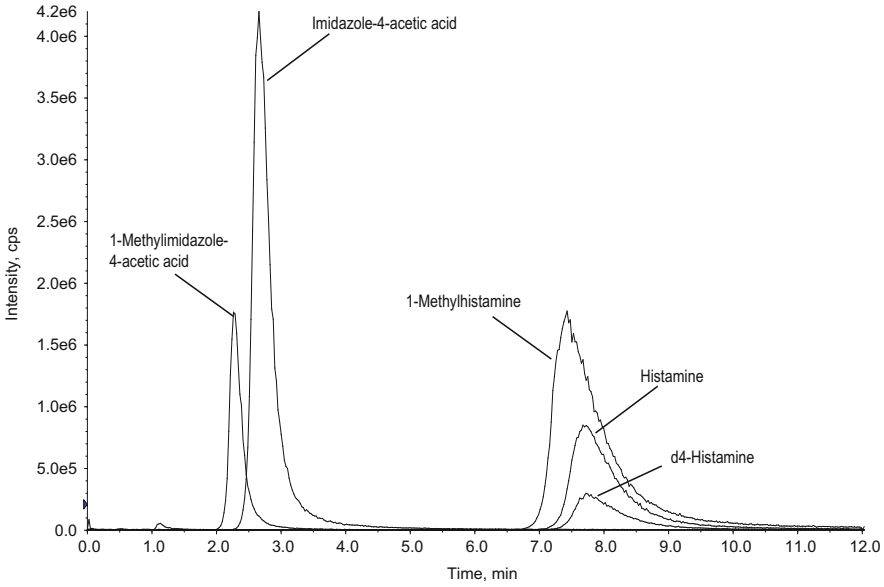

Fig. 3 Representative chromatogram of a calibrator containing histamine, 1-methylhistamine, imidazole-4-acetic acid, and 1-methylimidazole-4-acetic acid. The final concentrations of HA and its metabolites were 0.7 μM in water. D4-HA (0.2 μM) was included as internal standard. LC-MS/MS analysis was performed as described in Sect. 4.1 for murine brain extracts

This method is well-suited for quantification of the highly polar HA metabolites 1-MI4AA and I4AA. However, due to the late elution of HA and 1-MH from the HILIC column, broad peaks arise (Fig. 3). This leads to a diminished sensitivity for these analytes, which may exclude the applicability of this method for determination of HA and 1-MH in serum or plasma samples.

4.2 Mouse Serum

In the following section an optimized method for the quantification of HA and 1-MH in murine serum samples is described in more detail.

4.2.1 Preparation of Calibration Standards

Since HA and 1-MH are endogenous metabolites, calibrators were prepared in 50 mg/mL bovine serum albumin (BSA; Sigma) as surrogate matrix. For both analytes a calibration curve was constructed using eight calibration standards at a concentration range of 0.0078–4 μM. 50 μL aliquots were stored at −20 °C.

4.2.2 HA and 1-MH Extraction from Serum Samples and Calibrators

50 μL of serum sample or calibrator were treated with 200 μL extraction solvent consisting of a mixture of methanol and acetonitrile (50/50 [v/v]). After

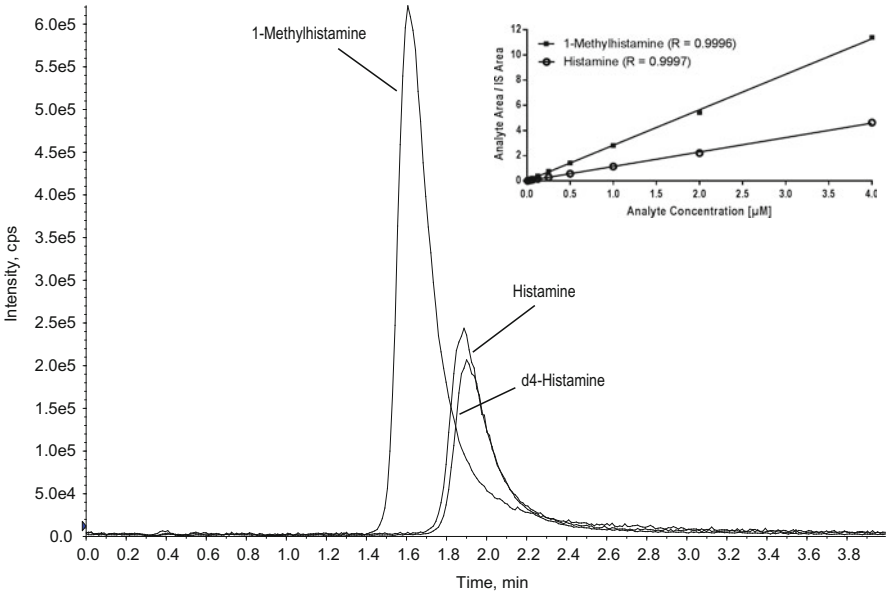

Fig. 4 Representative chromatogram of a calibrator containing histamine and 1-methylhistamine. HA and 1-MH calibrators were prepared in 50 mg/mL surrogate matrix. Analyte concentrations were 1.0 µM and the internal standard (d4-HA) concentration was 0.25 µM. LC-MS/MS analysis was performed as described in Sect. 4.2 for murine serum samples. Calibration curves for both analytes were constructed using linear regression and 1/x-weighing. Both calibrations curves show excellent correlation factors ($r > 0.999$). The lower limits of quantification for HA and 1-MH were 7.8 nM and 15.6 nM, respectively

centrifugation for 10 min at 20.800 × *g* and at 4 °C, the supernatant fluid was evaporated to dryness under a gentle nitrogen stream. The residue pellet was dissolved in 50 µL 80/20 acetonitrile/water [v/v] containing 0.25 µM of the internal standard d4-HA. 10 µL of this solution were injected into the HPLC system.

4.2.3 LC-MS/MS Conditions

Analysis of HA and 1-MH was achieved using LC-MS/MS. Chromatographic separation was performed using a Shimadzu system (Shimadzu, Duisburg, Germany), consisting of two HPLC-Pumps (LC-30AD), a temperature-controlled autosampler (SIL-30AC), a degasser (DGU-20A5), a column oven (CTO-20AC), and a control unit (CBM-20A). An EC Nucleodur 100-3 HILIC column (50 × 2 mm; 3 µm) equipped with a CC 8/3 Nucleodur HILIC Security Guard (3 µm) was purchased from Macherey-Nagel (Munich, Germany) and served as column for analyte separation in HILIC mode. Solvents A and B were 90/10 acetonitrile/water [v/v] (A) and 5/95 acetonitrile/water [v/v] (B), each containing 15 mM ammonium formate and 0.1% formic acid. For chromatographic separation the column was kept at 60 °C. An isocratic flow (600 µL/min) using 80% of solvent A was applied. The total run time was 4 min.

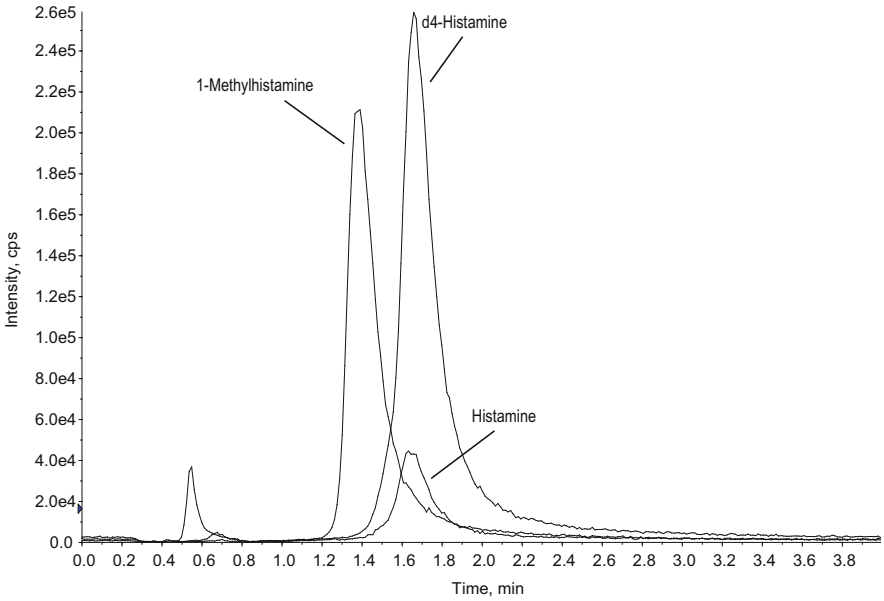

Fig. 5 Representative chromatogram of histamine and 1-methylhistamine from a murine serum sample. LC-MS/MS analysis was performed as described in Sect. 4.2 for murine serum samples. Concentration values of 0.14 µM (HA) and 0.24 µM (1-MH) were calculated

Detection and quantification of HA and 1-MH was carried out on a QTRAP® 5500 mass spectrometer (Sciex, Framingham, MA, USA) equipped with an electrospray ionization source, operating in positive ionization mode. For SRM detection, the following mass transitions were identified (see Table 2): HA: *m/z* 112 → 68 (quantifier) and *m/z* 112 → 95 (identifier); 1-MH: *m/z* 126 → 68 (quantifier) and *m/z* 126 → 109 (identifier). D4-HA (*m/z* 116 → 99) served as internal standard for HA as well as for 1-MH.

Control of the LC and MS/MS systems as well as data sampling was performed by Analyst software, version 1.5.2 (Sciex). Data interpretation of the MS/MS signals was carried out by calculating ratios of the peak areas of the calibrators and samples in relation to the respective peak areas of the internal standard.

Figure 4 shows a typical chromatogram of HA and 1-MH for a calibration standard (1 µM) including d4-HA (0.2 µM). Elution of HA and 1-MH occurred at 1.9 and 1.7 min, respectively. Constructed calibration curves (linear regression, 1/x) show excellent correlation ($r > 0.999$ for both analytes). The lower limits of quantification were 0.0156 µM for HA and 0.0078 µM for 1-MH.

With this method various murine serum sample were analyzed. A typical chromatogram is shown in Fig. 5. Calculated concentrations for HA and 1-MH were 0.14 µM and 0.24 µM, respectively. Furthermore, intensities of d4-HA in the calibration sample and in the serum sample are comparable, indicating that 50 mg/mL BSA is an adequate surrogate matrix for analyzing murine serum samples.

5 Conclusions and Perspectives

The main recommendations regarding critical steps in HA analysis by LC-MS/MS are (1) adequate sample preparation steps resulting in an instant stop of cellular metabolism and removal of interfering matrix components, (2) selection of robust and reproducible LC conditions, and (3) implementation of reliable MS/MS recordings. However, due to the comparatively low mass accuracy of triple quadrupole mass spectrometers, high resolution mass spectrometers should be additionally applied in HA research. The described LC-MS/MS methods for HA and its main metabolites can easily be upgraded with respect to further polar metabolites, such as various additional neurotransmitters.

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Structural Analysis of the Histamine H_1 Receptor

Mitsunori Shiroishi and Takuya Kobayashi

Contents

Abstract

The crystal structure of the human histamine H_1 receptor (H_1R) has been determined in complex with its inverse agonist doxepin, a first-generation antihistamine. The crystal structure showed that doxepin sits deeply inside the ligand-binding pocket and predominantly interacts with residues highly

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conserved among other aminergic receptors. This binding mode is considered to result in the low selectivity of the first-generation antihistamines for H_1R. The crystal structure also revealed the mechanism of receptor inactivation by the inverse agonist doxepin. On the other hand, the crystal structure elucidated the anion-binding site near the extracellular portion of the receptor. This site consists of residues not conserved among other aminergic receptors, which are specific for H_1R. Docking simulation and biochemical experimentation demonstrated that a carboxyl group on the second-generation antihistamines interacts with the anion-binding site. These results imply that the anion-binding site is a key site for the development of highly selective antihistamine drugs.

Keywords

Crystal structure • Doxepin • First-generation antihistamines • Histamine H_1 receptor • Receptor inactivation mechanism • Receptor selectivity of antihistamines

1 Introduction

Histamine H_1 receptor (H_1R), originally cloned from bovine H_1R (Yamashita et al. 1991), is a G protein-coupled receptor (GPCR) implicated in type I hypersensitivity allergic reactions caused by various kinds of allergens. H_1R is expressed in various tissues throughout body, including the airway, vascular smooth muscle, and brain (Hill 1990). Allergic reactions occur when allergens activate mast cells, which in turn release histamine and other bioactive substances. The released histamine then binds to and activates H_1R on surrounding vascular endothelial cells, causing vasodilation and vascular hyperpermeability, resulting in allergic inflammation. H_1R expressed in the brain plays an important role in the regulation of sleep-arousal cycle and memory by binding histamine as it acts as a neurotransmitter (Schwartz et al. 1991; Hill et al. 1997).

Antihistamines are first-line drugs for relief of allergic reactions and are inverse agonists of H_1R. Antihistamines suppress allergic reactions by stabilizing the equilibration of H_1R into its inactive state (Bakker et al. 2000). The long history of the development of antihistamines, and the fact that H_1R has one of the highest numbers of drugs approved to target its activity, illustrates the importance of this receptor (Overington et al. 2006). First-generation antihistamines have high blood-brain barrier permeability and low receptor selectivity, causing various side effects including drowsiness and dry mouth (Cusack et al. 1994). These days, second-generation antihistamines are developed, which significantly reduce brain permeability, although residual central nervous system effects are still remained (Tashiro et al. 2009). Challenges remain even for second-generation drugs, such as low affinity to the receptor and cardiotoxicity because of their interaction with cardiac potassium channels (Woosley et al. 1993; Yap and Gamm 2002). In this chapter, we

explain findings regarding the crystal structure of human H_1R complexed with its inverse agonist doxepin.

2 Stabilization and Production of H_1R Protein for Structural Study

The largest obstacle for the determination of a crystal structure is the preparation of milligram quantities of highly purified and stable receptor protein. To produce the receptor protein in a sufficient quantity and obtain high-quality crystals, an H_1R variant was constructed (Shiroishi et al. 2012). Human H_1R is difficult to overexpress owing to its very long third intracellular loop (ICL3), presumably because this long flexible region is a target of degradation and/or destabilizes the receptor in the cell. To overcome this difficulty, the lysozyme derived from T4 lysozyme (T4L), which was a successful fusion partner for structural determination of β2-adrenergic receptor (Rosenbaum et al. 2007), was fused onto the region of the i3-loop (Gln222-Gly404). This fusion had a significant effect not only on stabilizing the receptor but also on improving crystallization. Furthermore, the unstructured N-terminal 19 residues including a predicted N-linked glycosylation site were deleted, because heterogeneous glycosylation hampered crystallization. The H_1R variant was overexpressed in the methylotrophic yeast *Pichia pastoris* and purified in milligram quantities (Shiroishi et al. 2011). The H_1R variant expressed in yeast showed the same ligand-binding properties as the wild-type receptor. The purified receptor was crystallized in complex with its inverse agonist doxepin by the lipidic cubic phase (LCP) method. The diffraction data were collected using the synchrotron light source, and the structure was determined at 3.1 Å resolution (Shimamura et al. 2011).

3 Overall Structure of H_1R

As observed in the other GPCRs whose structures are known, H_1R consists of seven transmembrane (TM) helixes (TM1–TM7) and a short amphipathic helix (H8) (Fig. 1a). The intracellular loops (ICLs) and extracellular loops (ECLs) that connect the helixes were observed except for ICL3, because this ICL had been replaced with the fused T4L. The electron density of a portion (Phe168–Val174) of ECL2, which is the longest extracellular loop, could not be resolved, presumably because of its high degree of flexibility. The relative orientation of TM helixes of H_1R was similar to other inactive GPCR structures. Comparing the root mean square deviations (RMSDs) of 175 Cα atoms on seven TM helixes showed that the orientation of H_1R was most similar to the following aminergic receptors: β_1-adrenergic receptor (AR) (1.5 Å) (Warne et al. 2008), β_2-AR (1.3 Å) (Cherezov et al. 2007), and dopamine D_3 receptor (D_3R) (1.3 Å) (Chien et al. 2010). Larger deviations were observed for adenosine A_{2A} receptor ($A_{2A}R$) (2.3 Å) (Jaakola

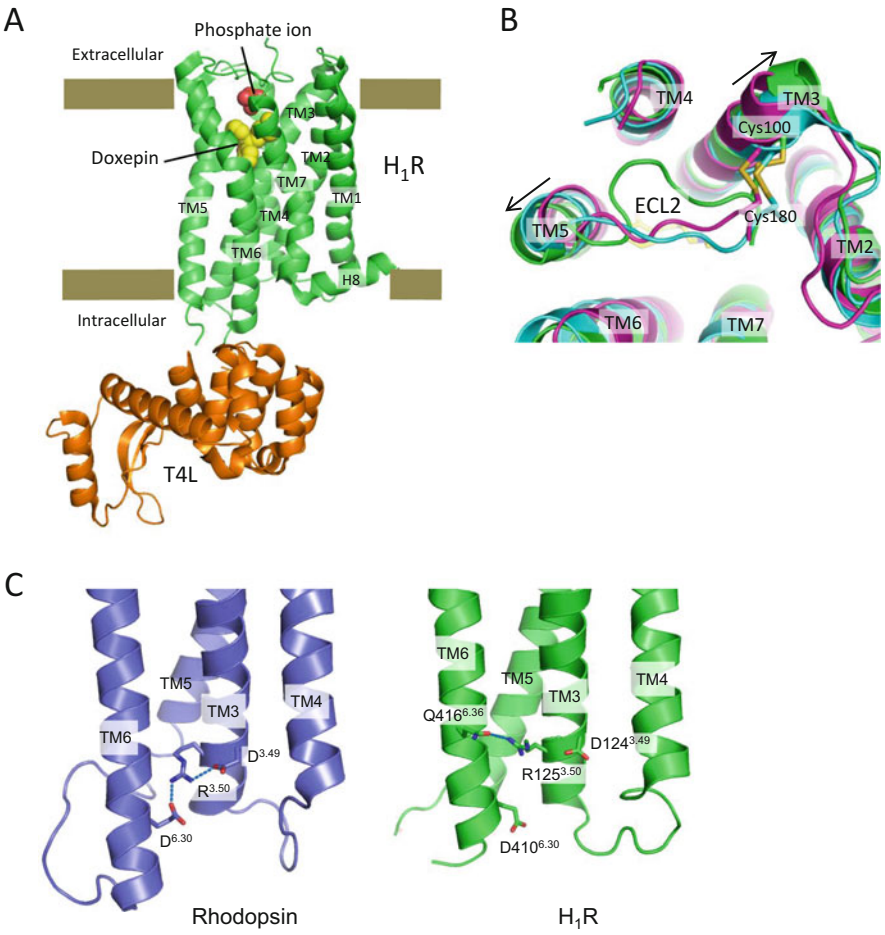

Fig. 1 (**a**) Overall structure of H_1R-doxepin complex. H_1R is represented by the *green* ribbon. Fused T4 lysozyme (T4L) replacing ICL3 is represented by the *orange* ribbon. Doxepin and phosphate ion are depicted as *yellow* and *orange* spheres, respectively. (**b**) Top view of the entrance of the ligand-binding pocket. H_1R, β_2-AR, and D_3R are superimposed and are represented by *green*, *cyan*, and *magenta* ribbons, respectively. The cysteine residues that form disulfide bond are shown as *yellow* sticks. (**c**) (*Left*) Ionic lock formation between $Arg^{3.50}$ and $Asp^{6.30}$ in rhodopsin. (*Right*) The structure of the side chains in the equivalent region in H_1R

et al. 2008) and CXCR4 chemokine receptor (2.2 Å) (Wu et al. 2010), which are more phylogenetically distant.

GPCR crystal structures have elucidated the structural diversity among their extracellular regions. In particular, ECL2 is the most variable region among GPCR receptors in amino acid sequence and structure. In H_1R, a disulfide bond conserved among many GPCRs was formed between Cys100 in the extracellular end of TM3 and Cys180 in ECL2 (Fig. 1b). This disulfide bridge anchored ECL2 to the entrance

of the ligand-binding pocket. Seven amino acids existed between the disulfide bridge and the extracellular end of TM5 in ECL2 in H_1R. This was greater than the number of amino acids found in this location in other aminergic receptors: β_2-AR has five amino acids at this location and D_3R has three. This seemed to extend the distance between the extracellular ends of TM3 and TM5 in H_1R compared to that in β_2-AR and D_3R, creating a larger space within the ligand-binding pocket (Fig. 1b).

A unique characteristic observed in the TM4 helix of H_1R is the kink induced by Pro161$^{4.59}$ (4.59 position of the Ballesteros-Weinstein numbering). Where an i + 4 turn is formed in β_2-AR and D_3R, a tighter i + 3 turn is formed in H_1R. This tighter turn allows accommodation of the bulky side chain of Trp158$^{4.56}$, where a serine is located in β_2-AR and D_3R. Trp158$^{4.56}$ is considered important for ligand specificity of aminergic GPCRs since mutations of this tryptophan to alanine, methionine, or phenylalanine reduce the affinity against the antagonist pyrilamine in the guinea pig H_1R (Wieland et al. 1999).

A D(E)RY motif in TM3 is well conserved among many GPCRs. Since the first observation of salt bridge formation between Arg$^{3.50}$ in this motif and Asp$^{6.30}$ in bovine rhodopsin, it has been suggested that this salt bridge (called an "ionic lock" in this context) stabilizes the inactive conformation (Palczewski et al. 2000). An ionic lock is observed in D_3R, but not in other GPCR structures. In H_1R, an ionic lock was not observed between Arg125$^{3.50}$ and Glu410$^{6.30}$ in the crystal structure (Fig. 1c). Instead, a hydrogen bond was formed between Arg125$^{3.50}$ and Gln416$^{6.36}$. This change in bond type could be induced by the fusion of T4L into ICL3. To reveal the structure of a possible ionic lock, determination of the structure without a fusion protein is necessary.

4 Structure of the Ligand-Binding Pocket and Low Selectivity of Doxepin

The inverse agonist doxepin consists of a tricyclic dibenzo[b,e]oxepin ring and an amine moiety, connected by a carbon chain (Fig. 2a). Doxepin was observed in the bottom of the ligand-binding pocket of the receptor (Fig. 1a). The lower side of the tricyclic ring sits in the ligand-binding pocket as deeply as the trimethyl-cyclohexane ring of retinal sits in rhodopsin, which is located deeper than the ligands in other GPCRs whose structures have been determined (Fig. 2b). As observed in the other aminergic receptors, the amino group of doxepin formed a salt bridge to Asp107$^{3.32}$ in TM3 (Fig. 2c). This salt bridge was found to be essential for binding both agonist and antagonist by mutational study (Bruysters et al. 2004; Nonaka et al. 1998; Ohta et al. 1994).

Doxepin, a first-generation antihistamine, binds to H_1R with very high affinity ($K_d = 0.69$ nM). However, due to its low receptor selectivity, it also binds to the other aminergic receptors with considerable affinities (serotonin receptor $5HT_{2A}$, 3.3 nM; muscarinic M_1 receptor, 6.8 nM; α_1-AR, 38 nM; D_2R, 63 nM) (Nonaka et al. 1998). The crystal structure of the H_1R-doxepin complex revealed that the

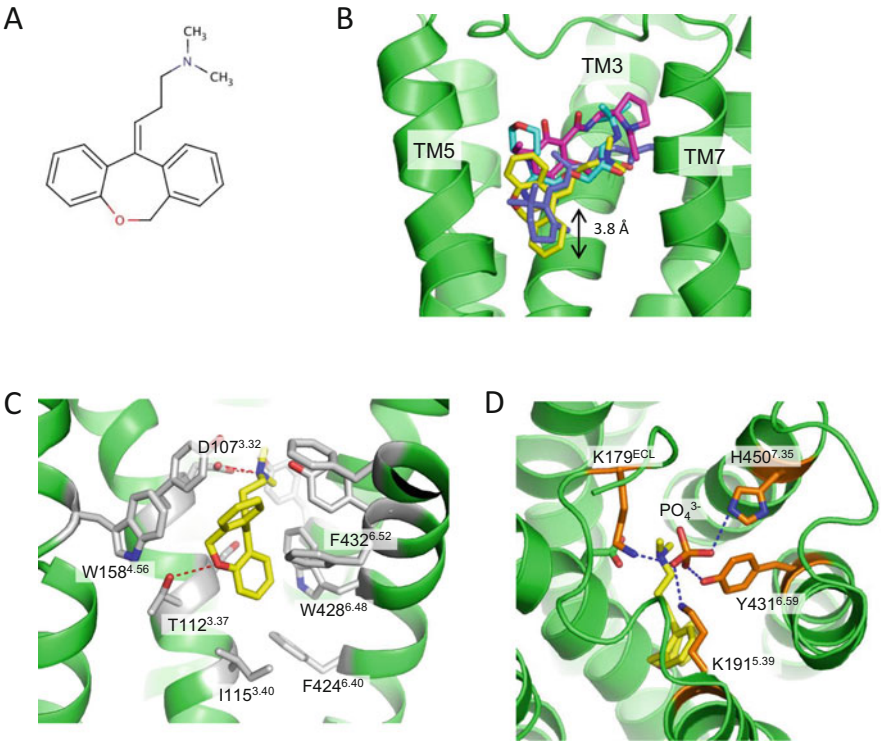

Fig. 2 (**a**) Chemical formula of doxepin. (**b**) The relative positions of the ligands bound in GPCRs. The coordinates of H_1R-doxepin complex, rhodopsin-retinal complex, β_2-AR-timolol complex (Hanson et al. 2008), and D_3R-eticlopride complex are superimposed, and H_1R is represented by the *green* ribbon. Doxepin, retinal, timolol, and eticlopride are shown as *yellow*, *purple*, *cyan*, and *magenta* sticks, respectively. TM6 is not shown for easier viewing. Doxepin bound more deeply compared to timolol, by 3.8 Å. (**c**) H_1R residues interacting with doxepin. H_1R is represented by the *green* ribbon. Doxepin and interacting residues are shown as *yellow* and *gray* sticks, respectively. Oxygen and nitrogen atoms are shown in *red* and *blue*, respectively. The salt bridge to Asp107 and hydrogen bond to T112 are represented by *red dashed lines*. (**d**) The phosphate-binding site of H_1R. H_1R is represented by the *green* ribbon. Phosphate ions and interacting residues are shown as *orange* sticks. Oxygen and nitrogen atoms are shown in *red* and *blue*, respectively. The electrostatic interactions are shown as *blue dashed lines*

tricyclic dibenzo[b,e]oxepin ring of doxepin interacts with hydrophobic residues in H_1R such as Ile112$^{3.37}$, Ile115$^{3.40}$, Phe424$^{6.44}$, Trp428$^{6.48}$, and Phe432$^{6.52}$(Fig. 2c). These residues are highly conserved among aminergic receptors (Table 1). Low receptor selectivity of doxepin is likely derived from the interactions with these highly conserved residues.

Table 1 Residues in the doxepin-binding site of H_1R and their equivalents in other aminergic receptors

	H_1R	H_2R	5-HT_{1A-F}	5-HT_{2A-C}	M_{1-5}	α_{1A-D}	α_{2A-C}	β_{1-3}	$D_{1,5}$	D_{2-4}
TM3	D107	D	D	D	D	D	D	D	D	D
	Y108	V	V/I/M	V	Y	V	V	V	I	V
	S111	C	C	S	S	C	C	V	S	C
	T112	T	T	T	N	T	T	T	T	T
	I115	I	I	I	I	I	I	I	I	I
TM4	W158	L	I	I	L	L	I	T	I	I/V
TM5	T194	D	S	G	T	S	S	S	S	S
	N198	T	A	S/A	A	S	S	S	S	S
TM6	F424	F	F	F	F	F	F	F	F	F
	W428	W	W	W	W	W	W	W	W	W
	Y431	Y	F	F	Y	F	F	F	F	F
	F432	F	F	F	F	F	F	F	F	F
	F435	F	A/S/E	N	V	M/L	Y	N	N	H
TM7	Y458	Y	Y	Y	Y	Y	Y	Y	W	Y

Abbreviations of receptors; *H_2R* histamine H_2 receptor, *5-HT_{1A-F}* serotonin 5-HT_{1A-F} receptors, *5-HT_{2A-C}* serotonin 5-HT_{2A-C} receptors, *M_{1-5}* M_{1-5} muscarinic acetylcholine receptors, *α_{1A-D}* α_{1A-D}-adrenergic receptors, *α_{2A-C}* α_{2A-C}-adrenergic receptors, *β_{1-3}* β_{1-3}-adrenergic receptors, *$D_{1,5}$* dopamine D_1 and D_5 receptors, *D_{2-4}* dopamine D_{2-4} receptors

5 Implications for the Development of Highly Specific Ligands of H_1R

Another notable characteristic of the H_1R-doxepin complex structure was the identification of an "anion-binding site" comprised of basic amino acids Lys191$^{5.39}$, Lys179^{ECL2}, and His450$^{7.35}$ on the extracellular portion of H_1R (Figs. 1a and 2d). A phosphate ion, which was present in the crystallization reservoir solution, was observed at the anion-binding site forming electrostatic interactions to the basic amino acids. The thermal stability of H_1R increased in the presence of phosphate ions even at physiological concentrations (~1.5 mM) (Shimamura et al. 2011). This indicates that the phosphate ion binds to the receptor at this site and serves as a positive modulator of ligand binding. Since the amino acids that form this anion-binding site are not conserved among other aminergic receptors, this site is unique to H_1R (Table 2).

Some second-generation antihistamines, showing higher specificity for H_1R, have a carboxyl group. The binding modes of these compounds to H_1R were predicted by molecular dynamics simulation in silico, showing that the carboxyl group of second-generation antihistamines sits in the anion-binding site. Furthermore, the thermal stability of H_1R complexed with the second-generation antihistamine cetirizine did not change in the presence of phosphate ion. Together, these results suggest that the carboxyl group of cetirizine sits in the anion-binding site and competes with the phosphate ion. Since the anion-binding site is unique to

Table 2 Residues in the phosphate-binding site of H_1R and their equivalents in other aminergic receptors

	H_1R	H_2R	5-$HT_{1A\text{-}F}$	5-$HT_{2A\text{-}C}$	$M_{1\text{-}5}$	$\alpha_{1A\text{-}D}$	$\alpha_{2A\text{-}C}$	$\beta_{1\text{-}3}$	$D_{1,5}$	$D_{2\text{-}4}$
ECL2	K179	K	A/E/D/Q	T/S	E/Q	I/E/F	R/Q	C	N	E/V
TM5	K191	G	T	V/M	T	V/A	V/I	A/V	A	V
TM6	Y431	Y	F	F	Y	F	F	F	F	F
	F435	F	A/S/E	N	V	M/L	Y	N	N	H
TM7	H450	E	F/G/A/S	L	W	F	F	F/Y	F	Y/V
	I454	L	N/T/A	V	Y	F	F	N	V	T

Abbreviations of receptors; *H_2R* histamine H_2 receptor, *5-$HT_{1A\text{-}F}$* serotonin 5-$HT_{1A\text{-}F}$ receptors, *5-$HT_{2A\text{-}C}$* serotonin 5-$HT_{2A\text{-}C}$ receptors, *$M_{1\text{-}5}$* $M_{1\text{-}5}$ muscarinic acetylcholine receptors, *$\alpha_{1A\text{-}D}$* $\alpha_{1A\text{-}D}$-adrenergic receptors, *$\alpha_{2A\text{-}C}$* $\alpha_{2A\text{-}C}$-adrenergic receptors, *β_{1-3}* $\beta_{1\text{-}3}$-adrenergic receptors, *$D_{1,5}$* dopamine D_1 and D_5 receptors, *$D_{2\text{-}4}$* dopamine $D_{2\text{-}4}$ receptors

H_1R, this could be a potential site for the development of antihistamines with higher specificity.

In recent years, structure-based virtual screening (SBVS), a computational screening method using a homology model of the target receptor, has been utilized as a strategy for rational drug screening. With the SBVS strategy using the crystal structure of H_1R, novel fragment-like compounds (less than 22 heavy atoms) with substantial affinities for H_1R ($K_d = 10$ μM–6 nM) have been successfully found with a high hit rate of 73% (de Graaf et al. 2011). These results highlight the importance of elucidating GPCR structures to assist in rational drug discovery.

6 Structural Insights into the Inverse Agonism of Doxepin

Since the active conformation of H_1R has not been crystallized, the activation mechanism is unclear. However, comparing known structures of active (agonist-bound) and inactive GPCRs has elucidated common features of GPCR activation. Translocation of several helices (TM1, TM3, TM5, TM6, and TM7) is found on the extracellular and intracellular side. In particular, TM3 and TM6 play a central role. It is speculated that the hydrophobic residues located in the core of the receptor, such as Ile/Leu/Val$^{3.40}$, Phe$^{6.44}$, and Trp$^{6.48}$, are involved in these movements (Tehan et al. 2014).

The CWxP$^{6.50}$ motif in TM6 is well conserved among family-A GPCRs and is one of the key molecular switches for activation of these receptors. Trp428$^{6.48}$, located in the bottom of the ligand-binding pocket, is called a "rotamer toggle switch" (or a "transmission switch"), because the ligand-induced shift of this residue results in a large rotation and movement of TM6 through rearrangement of the TM3-5-6 interface. This change in receptor configuration is essential for G protein activation upon agonist binding. This large structural change is observed in rhodopsin, $A_{2A}R$, and M_2R, but not in β_2-AR. In rhodopsin, retinal directly interacts with Trp$^{6.48}$ and stabilizes the receptor's inactive state. In the H_1R-doxepin structure, the tricyclic ring interacts extensively with Trp428$^{6.48}$, similarly to retinal's

interaction with inactive rhodopsin (Fig. 2c). This type of binding could stabilize the hydrophobic packing around TM6 and is unique among the known GPCR structures. The tricyclic ring of doxepin also interacts with $Ile115^{3.40}$ and $Phe424^{6.40}$, which are presumably involved in receptor activation. The ring moiety also interacts with $Ser111^{3.36}$, which acts as a toggle switch for activation upon agonist binding (Jongejan et al. 2005). The crystal structure of the H_1R-doxepin complex has clarified the interactions that stabilize H_1R in its inactive state, leading to large reduction of the basal activity of the receptor.

7 Conclusion

At present, X-ray crystallography is the only way to clarify ligand-receptor interaction at the atomic level. Over the past decade, the technologies of sample preparation, crystallization, and data collection have improved dramatically, and finally the crystal structure of H_1R was solved. The structure of the H_1R-doxepin complex provided critical information about receptor specificity of antihistamines and the mechanism of receptor inactivation. However, a number of points still remain to be clarified. For example, the resolution is not sufficient to understand the internal receptor-bound water molecules, which have recently been suggested to be important for receptor function (Sun et al. 2014). The structure of the activated form of H_1R also remains to be determined to delineate the activation mechanism. Moreover, the crystal structures of H_1R in complex with other antihistamines are needed to clarify the ligand specificity.

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Molecular Modelling Approaches for the Analysis of Histamine Receptors and Their Interaction with Ligands

Andrea Strasser and Hans-Joachim Wittmann

Contents

Abstract

Several experimental techniques to analyse histamine receptors are available, e.g. pharmacological characterisation of known or new compounds by different types of assays or mutagenesis studies. To obtain insights into the histamine receptors on a molecular and structural level, crystal structures have to be determined and molecular modelling studies have to be performed. It is widely

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accepted to generate homology models of the receptor of interest based on an appropriate crystal structure as a template and to refine the resulting models by molecular dynamic simulations. A lot of modelling techniques, e.g. docking, QSAR or interaction fingerprint methods, are used to predict binding modes of ligands and pharmacological data, e.g. affinity or even efficacy. However, within the last years, molecular dynamic simulations got more and more important: First of all, molecular dynamic simulations are very helpful to refine the binding mode of a ligand to a histamine receptor, obtained by docking studies. Furthermore, with increasing computational performance it got possible to simulate complete binding pathways of ions or ligands from the aqueous extracellular phase into the allosteric or orthosteric binding pocket of histamine receptors.

Keywords

Histamine receptors • Homology modelling • Molecular dynamics • Molecular modelling

Abbreviations

E2-loop	Extracellular loop E2
GPCR	G protein-coupled receptor
gpH_1R	Guinea-pig histamine H_1 receptor
$h5\text{-}HT_{1B}R$	Human serotonine 5-HT_{1B} receptor
$h5\text{-}HT_{2B}R$	Human serotonine 5-HT_{2B} receptor
hD_3R	Human dopamine D_3 receptor
hH_1R	Human histamine H_1 receptor
hH_2R	Human histamine H_2 receptor
hH_3R	Human histamine H_3 receptor
hH_4R	Human histamine H_4 receptor
hM_2R	Human muscarinic M_2 receptor
$h\beta_2R$	Human adrenergic β_2 receptor
MD	Molecular dynamics
MM	Molecular mechanics
QM	Quantum mechanics
QSAR	Quantitative structure activity relationship
$t\beta_1R$	Turkey adrenergic β_1 receptor
xH_xR	Different species of the four histamine receptor subtypes

1 Introduction

A large number of different experimental techniques to study histamine receptors, or in general GPCRs, are available: Ligands for example represent an important tool to characterise the receptors on a macroscopic level by different assays, e.g. radioligand competition binding assay, GTPase assay, luciferase assay or GTPγS binding assay (Seifert et al. 2013; Strasser et al. 2013; Panula et al. 2015). Furthermore, the resulting experimental data allow to analyse the histamine receptors also with regard to selectivity, e.g. species or subtype selectivity (Seifert et al. 2013; Strasser et al. 2013; Panula et al. 2015). However, these methods do not provide information about distinct ligand-receptor interactions or conformational changes of the receptor during the ligand binding or receptor activation process on a molecular level. Therefore, mutagenesis studies in combination with pharmacological characterisation are one important lab experimental method of choice (Kooistra et al. 2013; Seifert et al. 2013; Strasser et al. 2013; Schneider and Seifert 2016), because those studies give information about the influence of one or more amino acids onto the pharmacological properties of the analysed receptor, which have to be interpreted on a molecular level. In contrast, the determination of crystal structures of ligand-receptor complexes gives a detailed insight into the receptor conformation and the interactions between ligand and receptor (Venkatakrishnan et al. 2013). Although more and more crystal structures of aminergic GPCRs in the inactive and in the active state are available (Venkatakrishnan et al. 2013), (http://www.rcsb.org/, access date: 16.11.2016), this method is still limited to a small number of ligand-receptor complexes because of the high experimental expense. This gap between the pharmacological data on the one hand and the structural interpretation on a molecular level on the other hand can be closed with several molecular modelling approaches, as discussed later on in more detail. However, to improve the understanding of the histamine receptors for example with regard to species, subtype or functional selectivity, all these lab experimental and in silico techniques have to be used in a combined manner, as illustrated (Fig. 1) (Strasser 2009; Munk et al. 2016). Besides molecular modelling techniques, inclusive virtual screening methods are used for lead optimisation and identifying new affine histamine receptor ligands (Heifetz et al. 2016b; Levoin et al. 2016). Meanwhile, databases represent an important tool to improve the research in the GPCR field, because they provide a large amount of data, e.g. mutagenesis data, binding data or homology models (Southan 2016).

2 Molecular Modelling Approaches for Histamine Receptors

As illustrated in Fig. 1, a large number of different molecular modelling approaches to study GPCRs are available (Rodriguez et al. 2012; Costanzi 2013; Strasser and Wittmann 2013; Filizola 2014; Heifetz et al. 2016a). For most of these approaches, the structure of the GPCR is required. In the absence of X-ray structures, there were some attempts to model GPCRs de novo or ab initio, based on the amino acid

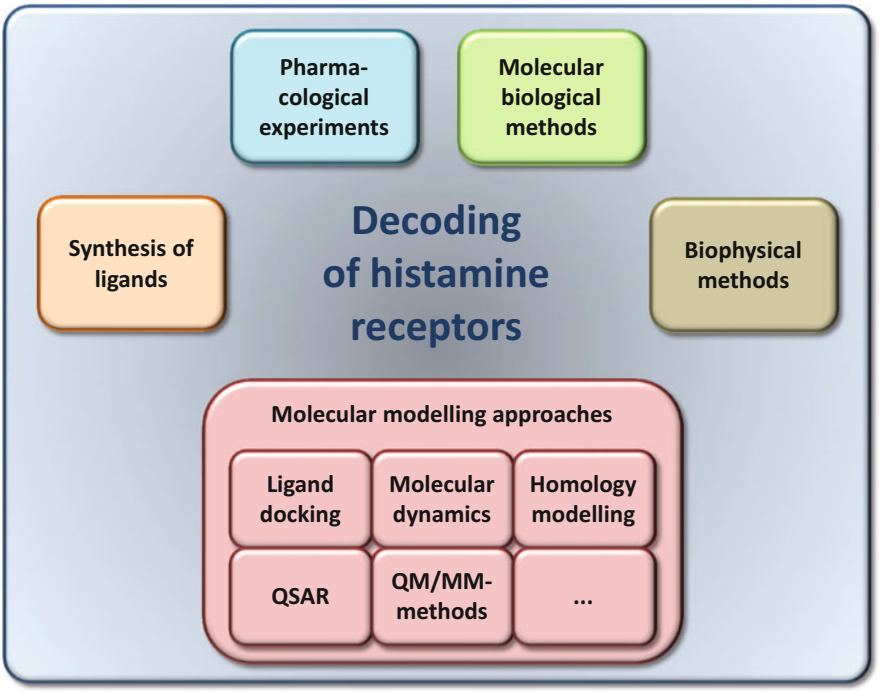


Fig. 1 Combination of experimental and molecular modelling techniques to obtain more detailed insight into the histamine receptors on a molecular level [modified according to Strasser (Strasser 2009)]

sequence (Filizola et al. 1999; de Graaf and Rognan 2009; Xu and Zhang 2012; Zhang et al. 2015). Although there are promising results with regard to those methods, they are not established so far. Nowadays it is state of the art to generate homology models of the GPCR of interest based on an appropriate crystal structure for the in silico analysis (de Graaf and Rognan 2009; Mobarec et al. 2009; Yarnitzky et al. 2010; Costanzi 2012; Koehler Leman et al. 2015).

2.1 Crystal Structure of the Histamine H_1 Receptor

Within the histamine receptors, only the crystal structure of the inactive human histamine H_1 receptor co-crystallised with the H_1R antagonist doxepin (pdb code: 3RZE, RCSB Protein Data Bank, http://www.rcsb.org/, access date: 16.11.2016) is available (Shiroishi and Kobayashi 2016; Shimamura et al. 2011). Thus, this crystal structure can be used, with some refinements, for in silico studies of the interactions between the human H_1R and antagonists.

2.2 Homology Models of Histamine Receptors

In the very beginning, homology models of histamine receptors based on the crystal structure of bacteriorhodopsin (Henderson et al. 1990) and later on crystal structures of bovine rhodopsin (Palczewski et al. 2000) were constructed (ter Laak et al. 1995; Bakker et al. 2004; Strasser and Wittmann 2007). Although the X-ray template used for homology modelling was not an aminergic GPCR, it was possible to explain pharmacological results quite well with those models (ter Laak et al. 1995; Bakker et al. 2004; Strasser and Wittmann 2007). However, the determination of crystal structures of aminergic GPCRs in its inactive state, available at the RCSB Protein Data Bank (http://www.rcsb.org/, access date: 16.11.2016) (Table 1), enables more appropriate templates for homology modelling of histamine receptors.

Furthermore, active-state homology models of H_xR-G-protein complexes, as recently described, for the hH_4R-Gαβγ-complex (Geyer et al. 2016) may also be generated, using the crystal structure of the $h\beta_2R$-Gαβγ-complex (pdb code: 3SN6) as a template (Rasmussen et al. 2011b).

The percentage of identical amino acids for the TM domains of the human histamine receptors and the aminergic GPCRs with a published crystal structure is given in Table 2. The percentage of identity of the single-TM domains ranges from ~12% up to 62%, whereas the overall identity of the TM domains ranges from ~27% up to ~42%. In general, the receptor with the highest homology to the receptor of interest should be used as a template (Fiser 2010). However, a threshold of at least 30% for accurate modelling of GPCRs is recommended (Fiser 2010). Within another approach, suggested to lead to improved results, different templates for different TM domains in homology modelling are used (Fiser 2010), even considering conserved inter-residue interactions (Chaudhari et al. 2015).

Table 1 Crystal structures of aminergic GPCRs in its inactive state, available at the PDB Protein Data Bank

GPCR	PDB code	Reference
$h\beta_2R$	2RH1, 2R4R, 2R4S, 3D4S, 3NYA, 3NY8, 3NY9, 3KJ6, 3P0G, 3PDS, 4GBR, 4LDE, 4LDL, 4LDO, 4QKX, 5D5A, 5D5B	(Cherezov et al. 2007; Rasmussen et al. 2007; Hanson et al. 2008; Bokoch et al. 2010; Wacker et al. 2010; Rasmussen et al. 2011a; Rosenbaum et al. 2011; Zou et al. 2012; Ring et al. 2013; Weichert et al. 2014; Huang et al. 2016)
$t\beta_1R$	2VT4, 2YCW, 2YCX, 2YCY, 2YCZ, 2Y00, 2Y01, 2Y02, 2Y03, 2Y04, 3ZPQ, 3ZPR, 4AMI, 4AMJ, 4BVN, 4GPO, 5F8U	(Warne et al. 2008; Moukhametzianov et al. 2011; Warne et al. 2011, 2012; Christopher et al. 2013; Huang et al. 2013; Miller-Gallacher et al. 2014; Leslie et al. 2015)
hD_3R	3PBL	(Chien et al. 2010)
h5-$HT_{1B}R$	4IAQ, 4IAR	(Wang et al. 2013)
h5-$HT_{2B}R$	4IB4	(Wacker et al. 2013)
hM_2R	3UON	(Haga et al. 2012)

Table 2 Percentage of identity between the amino acids of the human histamine receptors and aminergic GPCRs with a crystal structure published

	TM	$t\beta_1R$	$h\beta_2R$	hD_3R	$5\text{-}HT_{1B}R$	$5\text{-}HT_{2B}R$	hM_2R	hH_1R
hH_1R	1	20.0	30.0	23.3	30.0	26.7	26.7	100.0
	2	36.7	33.3	40.0	40.0	43.3	40.0	100.0
	3	39.4	36.4	33.3	39.4	39.4	42.4	100.0
	4	25.0	29.2	25.0	25.0	25.0	33.3	100.0
	5	26.5	17.6	29.4	32.4	17.6	32.4	100.0
	6	51.6	48.4	32.3	54.8	32.3	32.3	100.0
	7	57.1	47.6	61.9	52.4	33.3	47.6	100.0
	Σ1−7	36.0	34.0	34.0	38.9	31.0	36.0	100.0
hH_2R	1	33.3	26.7	33.3	26.7	26.7	26.7	33.3
	2	56.7	46.7	40.0	46.7	43.3	30.0	33.3
	3	48.5	42.4	60.6	48.5	48.5	42.4	36.4
	4	33.3	25.0	25.0	37.5	20.8	20.8	12.5
	5	32.4	38.2	29.4	29.4	26.5	26.5	29.4
	6	41.9	38.7	48.4	45.2	29.0	38.7	45.2
	7	61.9	38.1	57.1	47.6	42.9	42.9	52.4
	Σ1−7	43.3	36.9	41.9	39.9	34.0	32.5	34.5
hH_3R	1	40.0	33.3	36.7	33.3	33.3	23.3	20.0
	2	30.0	23.3	33.3	33.3	36.7	40.0	26.7
	3	33.3	27.3	33.3	30.3	39.4	39.4	36.4
	4	25.0	25.0	33.3	29.2	20.8	45.8	33.3
	5	26.5	20.6	26.5	14.7	17.6	14.7	17.6
	6	35.5	32.3	22.6	25.8	22.6	29.0	35.5
	7	42.9	33.3	47.6	33.3	33.3	33.3	33.3
	Σ1−7	33.0	27.6	32.5	28.1	29.1	31.5	28.6
hH_4R	1	33.3	40.0	26.7	30.0	30.0	20.0	16.7
	2	20.0	26.7	16.7	23.3	30.0	36.7	23.3
	3	36.4	36.4	39.4	39.4	39.4	45.5	39.4
	4	20.8	20.8	33.3	16.7	12.5	33.3	16.7
	5	14.7	8.8	20.6	17.6	17.6	20.6	20.6
	6	41.9	29.0	32.3	38.7	25.8	25.8	38.7
	7	33.3	38.1	38.1	33.3	38.1	38.1	38.1
	Σ1–7	28.6	28.1	29.1	28.6	27.1	31.0	27.6

The percentage of identity is given for the TM domains 1 (1.30–1.59), 2 (2.38–2.67), 3 (3.22–3.54), 4 (4.39–4.62), 5 (5.35–5.68), 6 (6.30–6.60) and 7 (7.33–7.53). The overall percentage of identity for all seven TM domains 1–7 is summarised as Σ1–7

However, in 2011, the crystal structure of the hH_1R in the inactive state in complex with the H_1R antagonist doxepin was published (Shimamura et al. 2011). A comparison of the homology model of hH_1R, based on the crystal structure of $h\beta_2R$ with the hH_1R crystal structure, showed that the homology model was in very good accordance to the X-ray structure of the hH_1R (unpublished results). Thus, carefully generated homology models represent the possibility to obtain detailed

insight into histamine receptors on a molecular level, if crystal structures are not yet solved, as for H_2R, H_3R and H_4R.

Although the transmembrane domains of GPCRs can be modelled in a good quality, it is a challenge to model loops or termini, e.g. the E2-loop or the N-terminus (Goldfeld et al. 2011; Arora et al. 2016). In a large number of the crystal structures, the E2-loop and the N-terminus are not or not completely solved. Thus, several tools have to be used to model those domains, offering a large number of different conformations. It was shown by several mutagenesis studies in combination with pharmacological analysis that amino acids of the E2-loop have influence onto affinity, potency and efficacy of ligands at the histamine receptors (Lim et al. 2008; Strasser et al. 2008b; Brunskole et al. 2011; Peeters et al. 2011; Wifling et al. 2015a). Thus, the correct modelling of the E2-loop is essential for a highly predictive homology model. However, the modelling of the loop regions, especially the E2-loop, remains quite challenging (Goldfeld et al. 2011; Arora et al. 2016). This is also reflected by comparison of the E2-loops of crystal structures of aminergic GPCRs (Fig. 2). Compared to the TM domains, the parts of the E2-loop, not being fixed by a disulphide bond, show a very high flexibility (Fig. 2a). While there are only small differences between the transmembrane

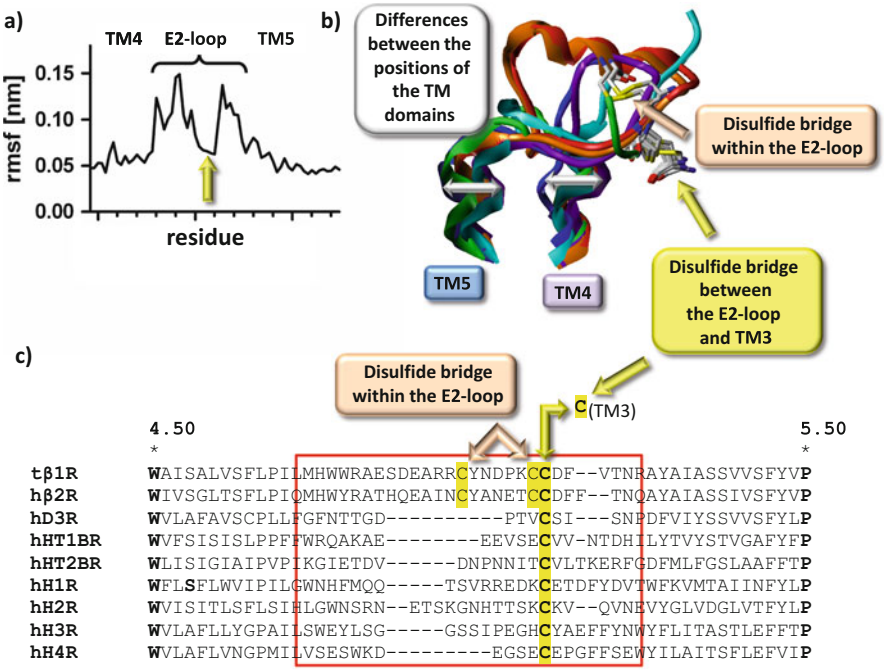


Fig. 2 E2-loop: (**a**) Flexibility, (**b**) differences in conformation (*orange*: $t\beta_1R$, *red*: $h\beta_2R$, *blue*: hD_3R, *violet*: h5-$HT_{1B}R$, *cyan*: h5-$HT_{2B}R$, *green*: hH_1R) and (**c**) differences in the amino acid sequences

domains, for the E2-loop very different conformations were found for the different receptors (Fig. 2b).

Another problem in modelling of E2-loops arises from the differences in length of the loops (Fig. 2c). However, the $t\beta_1R$ and the $h\beta_2R$ represent no appropriate template for modelling of the E2-loop of the histamine receptors, because within the E2-loop of the $t\beta_1R$ and $h\beta_2R$, an additional disulphide bridge, forcing a part of the E2-loop into a helical conformation, is present (Fig. 2b, c). Due to the lack of two additional cysteines, this second disulphide bridge is missing in the E2-loops of the histamine receptors (Fig. 2c). The number of amino acids between TM4 and the highly conserved cysteine ranges from 11 to 18 in the hH_xRs, but in the X-ray templates only 10 to 12 amino acids are present. Furthermore, the number of amino acids between the highly conserved cysteine of the E2-loop to TM5 ranges from 4 to 6 for the hH_xRs, while it ranges from 3 to 6 for the X-ray templates (Fig. 2c). The different lengths of the E2-loops may lead to differences in the extracellular positions of TM4 and TM5, as illustrated (Fig. 2b), and have also to be considered in homology modelling. Due to these differences not only in the chemical nature of the amino acids itself, but also in the length of the parts of the loops, it is highly challenging to model an appropriate conformation of the E2-loop. Furthermore, it has to be considered that more than one conformation of the E2-loop of the receptor may exist. Instead, it has to be speculated that an E2-loop can exhibit different conformations, e.g. in dependence of the ligand bound. However, the conformation of loops can be refined

Table 3 A small selection of useful databases and webservers for GPCR modelling (access date: 16.11.2016)

Name	URL	Comment
GPCR network	http://gpcr.usc.edu/	News in GPCR research, especially regarding the progress in crystallisation of a GPCR
GPCRdb	http://www.gpcrdb.org	Contains data (e.g. structures and mutation data) and Web tools for GPCRs
PDB	http://www.rcsb.org/	Contains data regarding experimentally determined structures of proteins
gpDB	http://biophysics.biol.uoa.gr/gpDB/	A database of GPCRs, G proteins, effectors and their interactions
GPCR-OKB	http://www.gpcr-okb.org/	A database regarding GPCR oligomerisation
Clustal	http://www.clustal.org	A software/Web server for multiple sequence alignment
I-TASSER	http://zhanglab.ccmb.med.umich.edu/I-TASSER/	Prediction of protein structures
PSIPRED	http://bioinf.cs.ucl.ac.uk/psipred	A server for prediction of protein structures
GPCR-ModSim	http://open.gpcr-modsim.org/	Modelling and simulation of GPCRs
GOMoDo	http://molsim.sci.univr.it/cgi-bin/cona/begin.php	A server for GPCR modelling and docking

by molecular dynamic simulations, taking into account the surrounding water molecules and ions, e.g. Na^+ and Cl^- (Arora et al. 2016).

Homology models of GPCRs can be generated manually, but meanwhile an increased number of servers and databases (Table 3) (Rodriguez et al. 2012; Koehler Leman et al. 2015) offer already prepared homology models or generate homology models. But due to the problems regarding the conformations of the loops and termini, in most cases only the TM domains are offered by servers or databases (Rodriguez et al. 2012).

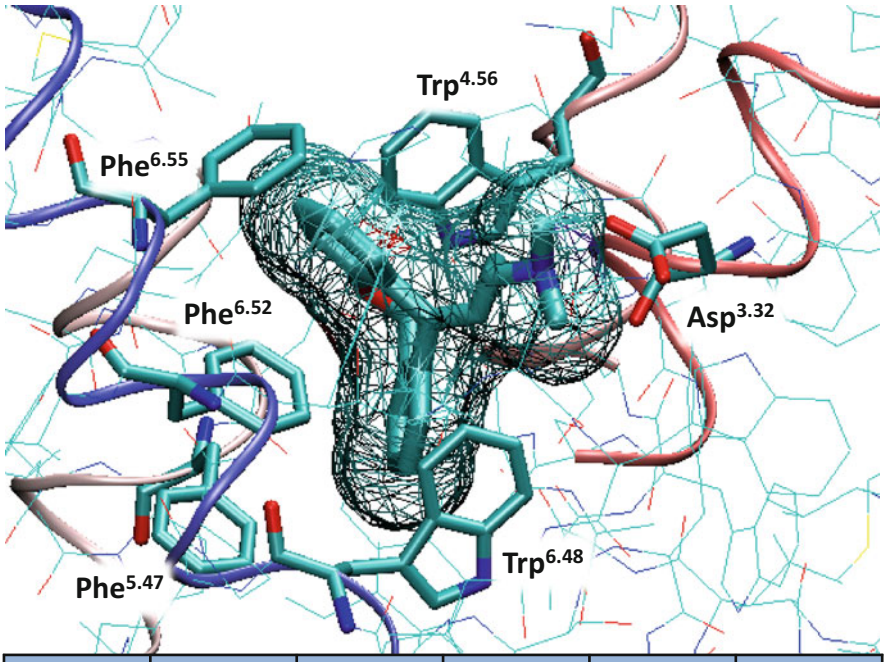
2.3 Different Modelling Techniques

2.3.1 QSAR

Quantitative structure-activity relationships (QSAR) are a method to describe the relation between the ligand structure and the pharmacological property, e.g. affinity, potency or even efficacy quantitatively (Verma et al. 2010; Cherkasov et al. 2014; Damale et al. 2014). A QSAR study requires a library of ligands with high structural similarity and which bind to the same binding site of the target. Furthermore, the compounds of the library have to be separated into a training set and a test set. The training set, necessary to calculate the quantitative structure-activity relationships, should contain at least 20 or 30 compounds, with known pharmacological parameters, e.g. affinity in the range of at least two orders of magnitude. The test set, necessary to analyse the quality of the QSAR model, should contain at least ten compounds, with the same pharmacological parameter, determined experimentally under the same conditions. Of course, these requirements limit the use of QSAR methods, which can be classified as retrospective methods. However, QSAR-based methods may represent a fast tool to understand the biological effect of drugs or to predict pharmacological parameters of compounds, also in the field of histamine receptors (Strasser 2009; Istyastono et al. 2011; Sirci et al. 2012; Kooistra et al. 2014).

2.3.2 Docking

Automated docking of ligands into the binding pocket of a GPCR is a very fast method to obtain one or more suggestions for the binding mode of a ligand (Beuming and Sherman 2012; Sandal et al. 2013; Beuming et al. 2015; Yuriev et al. 2015; Irwin and Shoichet 2016). Within modern docking routines rotatable bonds of the ligand and additionally of the amino side chains of the receptor are considered, leading to improved docking results, but also to increased computational costs. Although those methods were often successfully used to describe ligand-receptor interactions or to obtain starting structures for MD simulations (Strasser 2009; Schultes et al. 2013; Darras et al. 2014; Naporra et al. 2016), one has to be aware that such methods do not consider translational or rotational movements of the backbone. Consequently, differences in receptor conformation in dependence of the bound ligand cannot be investigated. Furthermore, these methods do not provide any information about the stability of the resulting ligand-receptor complex on the time course. A large number of studies suggest



Asp3.32								Trp4.56								Phe5.47							
apolar	aromatic (a)	aromatic (b)	H-bond (c)	H-bond (d)	Ionic interaction (e)	Ionic interaction (f)		apolar	aromatic (a)	aromatic (b)	H-bond (c)	H-bond (d)	Ionic interaction (e)	Ionic interaction (f)		apolar	aromatic (a)	aromatic (b)	H-bond (c)	H-bond (d)	Ionic interaction (e)	Ionic interaction (f)	
1	0	0	0	1	0	1		1	0	1	0	0	0	0		1	0	0	0	0	0	0	

Trp6.48								Phe6.52								Phe6.55							
apolar	aromatic (a)	aromatic (b)	H-bond (c)	H-bond (d)	Ionic interaction (e)	Ionic interaction (f)		apolar	aromatic (a)	aromatic (b)	H-bond (c)	H-bond (d)	Ionic interaction (e)	Ionic interaction (f)		apolar	aromatic (a)	aromatic (b)	H-bond (c)	H-bond (d)	Ionic interaction (e)	Ionic interaction (f)	
1	0	1	0	0	0	0		1	0	1	0	0	0	0		1	0	1	0	0	0	0	

(a) face to face
(b) edge to face
(c) receptor: donor; ligand: acceptor
(d) receptor: acceptor; ligand: donor
(e) receptor: positive; ligand: negative
(f) receptor: negative; ligand: positive

Fig. 3 Fingerprint for the binding mode of the H_1R antagonist doxepin in the hH_1R [modified according to Kooistra et al. (2016)]

that water molecules are present in the binding pocket, and even stabilise the interaction between ligand and receptor (Wagner et al. 2011; Kuhne et al. 2016). But within most docking studies, water molecules are in general not taken into account. Thus, docking results may give a first idea about a binding mode of a ligand, but should be refined by subsequent molecular dynamic studies, including approximately the physiological surrounding.

2.3.3 Molecular Interaction Fingerprint Methods

Within the computer-aided development of drugs, the data processing of a large amount of, e.g., docking results is a great challenge (Mordalski et al. 2011). To manage those data, e.g. H-bond or aromatic interactions within reasonable time, the

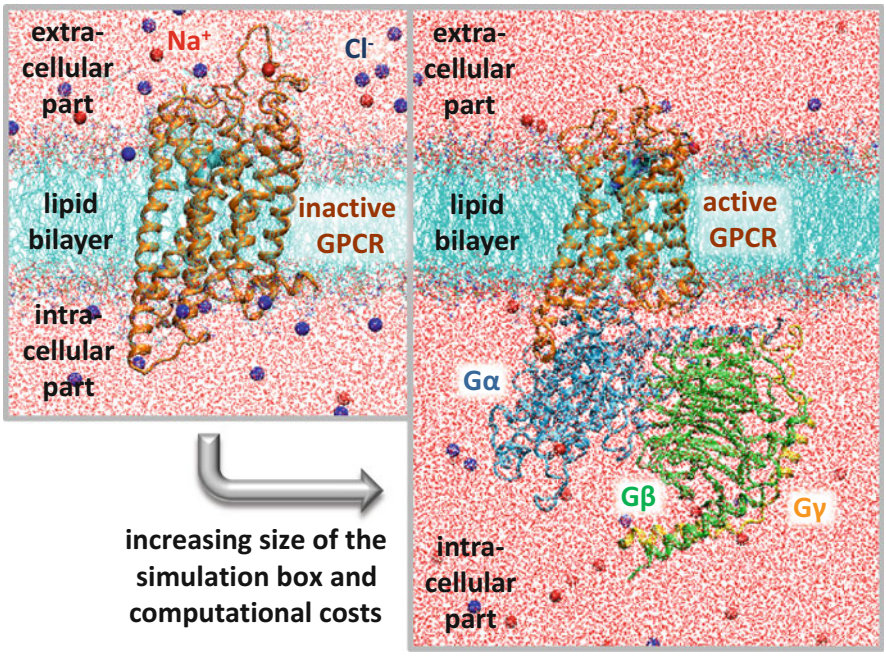


Fig. 4 Simulation boxes for a histamine receptor in its inactive state (*left*) and in its active state in complex with the Gαβγ-subunit (*right*)

structural or molecular interaction fingerprint approach was established (Deng et al. 2004; Mordalski et al. 2011; Vass et al. 2016), as shown exemplary for the binding mode of doxepin to the hH_1R (Fig. 3) (Kooistra et al. 2016). Those fingerprint methods were shown to be helpful, e.g. in prediction of binding modes or even functional activity (Vass et al. 2016).

2.3.4 Molecular Dynamic Simulations

A typical simulation box for a GPCR embedded in its lipid bilayer, being surrounded by water molecules and ions in approximately physiological concentrations, contains in general more than 50,000 sites (Fig. 4). If the Gαβγ complex is included in the simulation, the simulation box contains more than 200,000 sites.

Although MD simulations (Dror et al. 2012; Ciancetta et al. 2015; Tautermann et al. 2015; McRobb et al. 2016) are a very powerful and important tool to study conformational changes of the receptor or ligand-receptor complexes, the computational time, which increases exponentially with the number of sites in the simulation box, is a crucial point. The rotation around bonds takes place in the ps scale, whereas ion transport or ligand binding is ranged in general in the ns or μs scale (Selent et al. 2010; Dror et al. 2011; Yuan et al. 2011; Wittmann and Strasser 2015; Thomas et al. 2016). Furthermore, protein folding or large conformational changes within proteins are estimated to be in the ms scale.

Table 4 Comparison of the most important modelling techniques

Molecular modelling technique	Advantages	Disadvantages
QSAR	– Fast – High throughput	– A training set of at least 30 (similar) compounds with known pharmacological data, determined under the same experimental conditions required, in an affinity range of at least two orders of magnitude – Good predictive quality, if the compounds are similar to the compounds of the training set – Considering of flexibility (receptor, ligand) not possible – Considering of water molecules rather not possible
Rigid docking	– Fast – High throughput – No training set required	– Flexibility of receptor and ligand in the binding pocket not considered – Effects of specific solvation cannot be monitored – Conformational changes of the receptor cannot be considered
Flexible docking	– No training set required – Different minima can be detected	– High computational costs – Effects of specific solvation cannot be monitored – Conformational changes of the receptor cannot be considered
Molecular dynamics	– No training set required – Conformational changes of the ligand and receptor can be monitored – Water can enter into the binding pocket – Different minima can be deduced – Monitoring the time-dependent evolution of the system	– High up to very high computational costs in dependence of the simulation time – Small throughput – Breaking/forming of bonds is not possible
QM or QM/MM methods	– Breaking/forming of bonds is possible	– Very high computational costs – Small throughput

Nowadays it is possible to perform MD simulations of a typical GPCR simulation box (Fig. 4) up to some μs (Selent et al. 2010; Dror et al. 2011; Yuan et al. 2011; Thomas et al. 2016). Thus, it should be possible to observe conformational changes of the ligand in the binding pocket, and furthermore the binding of water or ions into the binding pocket or some conformational changes of the receptor. However, due to the limitation in computational time, it is not possible up to now to observe the whole binding process of an agonist and the subsequent activation of the receptor.

2.3.5 Semi-empirical Calculations, Ab Initio Methods, QM/MM Methods

Although molecular dynamics simulations are an important tool to study histamine receptors in dependence of time, it is not possible to monitor breaking or forming of bonds. Due to the high computational costs, linked with quantum mechanical methods, e.g. semi-empirical calculations or ab initio calculations, such calculations are rarely performed in the field of histamine receptors (Kovalainen et al. 2000; Jongejan et al. 2008). An accepted alternative are combined quantum mechanical and molecular mechanical (QM/MM) methods: Here, only a small part of the receptor, e.g. the binding pocket with bound ligand, is investigated on a quantum mechanical basis, whereas the remaining larger part of the system (receptor, surrounding) is investigated on a molecular mechanical basis.

2.3.6 Comparison of Different Modelling Techniques in the Histamine Receptor Research

As described above, a lot of different molecular modelling techniques to analyse histamine receptors on a molecular level are available (Table 4). QSAR or docking methods are quite fast with low computational costs, compared to quantum mechanical calculations or simulations. On the other hand, MD simulations allow to analyse a wide area of the conformational space of histamine receptors. To solve a distinct modelling problem, a skilful combination of stationary (QSAR, docking) and dynamic (MD) methods is essential.

3 Comparison of the Orthosteric and the Allosteric Binding Site of the Four Human Histamine Receptor Subtypes Based on the Amino Acid Sequence

The analysis of the amino acid sequence alignment of the four human histamine receptor subtypes (Seifert et al. 2013; Strasser et al. 2013) shows a homology within the TM helices between ~27 and ~54%. The largest homology is found between the hH_3R and hH_4R, while the smallest homology is found between hH_1R and hH_4R as well as between hH_2R and hH_4R.

However, the differences in pharmacological profiles of several ligands between the four histamine receptor subtypes are in general not a consequence of the overall differences in the amino acid sequence, but rather of the differences in amino acids of the binding pocket. Based on several crystal structures of aminergic GPCRs with a bound ligand (see Sect. 2.2), the orthosteric binding pocket is known quite well. However, to obtain information if a distinct amino acid is directly or indirectly involved in ligand binding, experimental mutagenesis studies with subsequent pharmacological investigation are performed. These experimental data provide an important input for molecular modelling studies, e.g. for refinement of present models. Hundreds of mutations were analysed within the subfamily of aminergic GPCRs (http://www.gpcrdb.org, access date: 16.11.2016). However, also at histamine receptors, a large number of mutagenesis studies were performed

(http://www.gpcrdb.org, access date: 16.11.2016) (Kooistra et al. 2013; Strasser et al. 2013). But not all amino acids are involved in the ligand binding. Only those amino acids close to the orthosteric (Fig. 5) binding site may have an influence on ligand binding.

The most important amino acids of the transmembrane domains, shown to be involved in ligand binding at the histamine receptors, are summarised in Fig. 6.

A comparison of the percentage of identical amino acids of the orthosteric binding site, suggested being involved in ligand binding, shows the highest homology of 69.2% for the hH_3R–hH_4R. All other pairs have a clearly smaller homology in the range from 30.8 to 38.5%. This explains that a large number of ligands, e.g. thioperamide or UR-PI294 (N^1-[3-(1*H*-imidazol-4-yl)propyl]-N^2-propionylguanidine), have affinity to hH_3R and hH_4R (Seifert et al. 2013). Additionally, the extracellular domains have influence on affinity, potency and efficacy for selected ligands, as shown, e.g., for the H_4R (Brunskole et al. 2011; Wifling et al. 2015b). As already mentioned, the extracellular domains, especially the E2-loop, show a very high flexibility. Thus, the prediction of amino acids of the extracellular domains being involved in ligand binding is quite a challenge (Goldfeld et al. 2011; Arora et al. 2016). Even if the influence is known by a combination of experimental mutagenesis and pharmacological studies, it is often not possible to explain the pharmacological data in a satisfactory manner, especially if extracellular domains are involved (Brunskole et al. 2011).

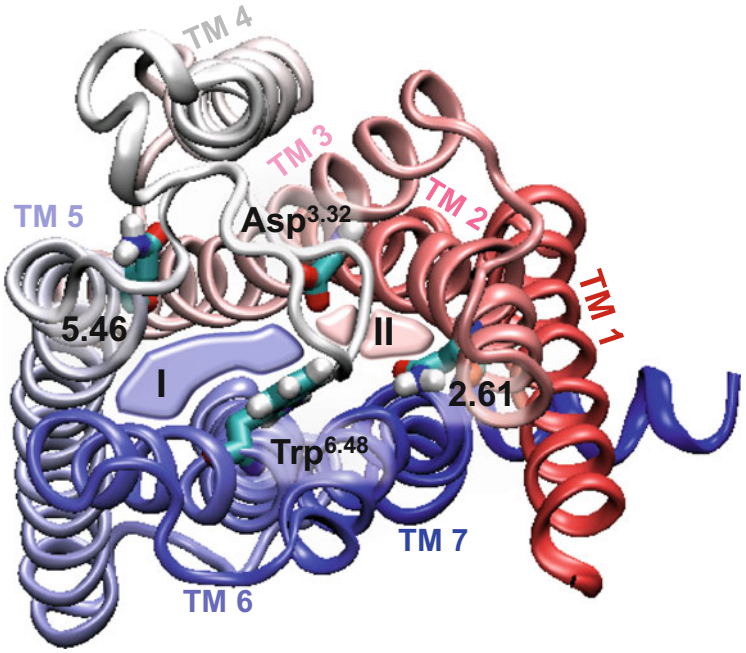
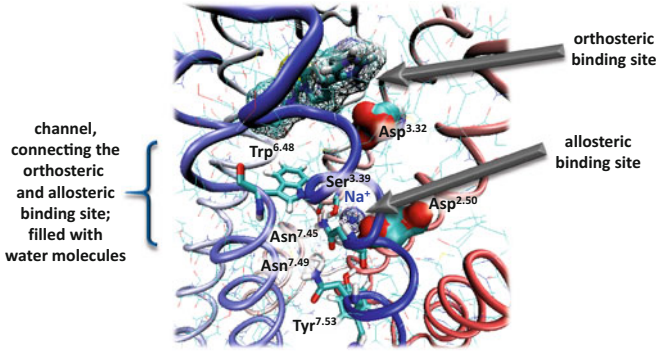


Fig. 5 Schematics of the orthosteric binding site with the main pocket I and the side pocket II. $Asp^{3.32}$ and $Trp^{6.48}$ are conserved within the histamine receptors; the amino acids at 2.61 and 5.46 differ between the histamine receptors and may be involved in species or subtype differences

Besides the orthosteric ligand-binding site, the allosteric binding site near to $Asp^{2.50}$ plays an important role for the binding of Na^+ or other monovalent cations, as described in more detail in Sect. 4.6. It was shown by mutagenesis studies or X-ray structures for several different GPCRs that the highly conserved $Asp^{2.50}$ acts as a binding site for Na^+ (Katritch et al. 2014; Strasser et al. 2015). Furthermore, within the allosteric ion-binding site, the amino acids $Asp^{2.50}$, $Ser^{3.39}$, $Asn^{7.45}$, $Ser^{7.46}$ and $Asn^{7.49}$, which are involved in binding of the Na^+, are highly conserved within class A of the GPCRs (Katritch et al. 2014; Strasser et al. 2015). These amino acids are also present within the four human histamine receptor subtypes (Fig. 6). However, a comparison of the most important amino acids, forming the allosteric binding site and the channel, connecting the orthosteric and allosteric site, shows that about 30% of the amino acids are different within the four human histamine receptor subtypes (Fig. 6), which may explain the differences in sodium sensitivity, e.g. between the hH_3R and hH_4R (Schneider et al. 2009; Schnell and Seifert 2010).



orthosteric binding site															
	2.61	3.32	3.33	3.36	3.37	3.40	5.38	5.39	5.42	5.43	5.46	6.48	6.51	6.52	7.42
hH_1R	Asn	Asp	Tyr	Ser	Thr	Ile	Phe	Lys	Thr	Ala	Asn	Trp	Tyr	Phe	Gly
hH_2R	Ser	Asp	Val	Cys	Thr	Ile	Tyr	Gly	Asp	Gly	Thr	Trp	Tyr	Phe	Gly
hH_3R	Tyr	Asp	Tyr	Cys	Thr	Ala	Phe	Leu	Ala	Ser	Glu	Trp	Tyr	Thr	Leu
hH_4R	Tyr	Asp	Tyr	Cys	Thr	Val	Ile	Leu	Thr	Ser	Glu	Trp	Tyr	Ser	Gln
allosteric (Na^+) binding site (and channel between the orthosteric and allosteric site)															
	2.46	2.49	2.50	3.35	3.36	3.39	6.44	6.48	7.42	7.43	7.45	7.46	7.49	7.50	7.53
hH_1R	Leu	Ala	Asp	Ala	Ser	Ser	Phe	Trp	Gly	Tyr	Asn	Ser	Asn	Pro	Tyr
hH_2R	Leu	Thr	Asp	Leu	Cys	Ser	Phe	Trp	Gly	Tyr	Asn	Ser	Asn	Pro	Tyr
hH_3R	Leu	Ser	Asp	Leu	Cys	Ser	Phe	Trp	Leu	Trp	Asn	Ser	Asn	Pro	Tyr
hH_4R	Leu	Ser	Asp	Leu	Cys	Ser	Phe	Trp	Gln	Trp	Asn	Ser	Asn	Pro	Tyr

Fig. 6 The orthosteric ligand and allosteric Na^+-binding site and the most important amino acids forming both sites of the human histamine receptors (*blue*: negatively charged, *red*: positively charged, *orange*: polar, *yellow*: cysteine, *green*: aromatic and polar, *dark grey*: aromatic and lipophilic, *grey*: lipophilic and bulky, *light grey*: lipophilic and small)

4 Molecular Modelling of Histamine Receptors: Impact for Understanding the Histamine Receptors on a Molecular Level: Case Studies

4.1 Binding Mode of Histamine at the Four Human Histamine Receptor Subtypes

The binding pocket of the histamine receptors is well characterised by mutagenesis studies (Kooistra et al. 2013): It was shown by mutagenesis studies that $Asp^{3.32}$, $Lys^{5.39}$, $Thr^{5.42}$, $Asn^{5.46}$, $Phe^{6.52}$ and $Phe^{6.55}$ have an influence on affinity and/or potency of histamine to the H_1R. The amino acids $Asp^{3.32}$, $Asp^{5.42}$ and $Thr^{5.46}$ were shown to be involved in binding of histamine to the hH_2R. Mutagenesis studies at the H_3R showed that $Leu^{5.39}$ has only small influence on affinity of histamine, whereas $Ala^{5.42}$ and especially $Glu^{5.46}$ have an influence on affinity of histamine. Furthermore, it was shown experimentally that the amino acids $Asn^{4.57}$, $Thr^{5.42}$, $Ser^{5.43}$, $Ser^{6.52}$ and especially $Glu^{5.46}$ are involved in binding of histamine to the H_4R.

To obtain a more detailed insight of the binding mode of histamine to the four histamine receptor subtypes on a molecular level, histamine was docked, considering experimentally determined mutagenesis and in silico data (Jongejan et al. 2005, 2008; Kooistra et al. 2013), into the orthosteric binding sites of the receptors (Fig. 7).

4.2 Binding Pathway of the Endogenous Ligand/Agonist Histamine to the Human Histamine H_4 Receptor

As described above, within several studies, the binding mode of histamine at the hH_4R was studied in silico by docking the histamine into the orthosteric binding site (Jongejan et al. 2008; Kiss et al. 2008). Although these studies are important, to interpret the results of mutagenesis studies on a molecular level, they give no information about the binding pathway of a ligand into its binding pocket of the receptor. However, in a recent study, the binding pathway of histamine into the orthosteric binding pocket of the hH_4R was observed by unconstrained molecular dynamic simulations and could be divided into four phases (Fig. 8) (Wittmann and Strasser 2015).

After a diffusion phase of the ligand in the aqueous phase (phase I, Fig. 9), a subsequent binding onto the extracellular surface of the hH_4R was observed (phase II, Fig. 9). Afterwards, the histamine bound rapidly (<1 ns) into the orthosteric binding pocket (phase IIIa, Fig. 9), followed by an orientation phase of the histamine in the orthosteric binding pocket (phase IIIb, Fig. 9) (Wittmann and Strasser 2015). During the binding process, negatively charged amino acids at the surface or within the binding channel between the extracellular surface and the orthosteric binding pocket were

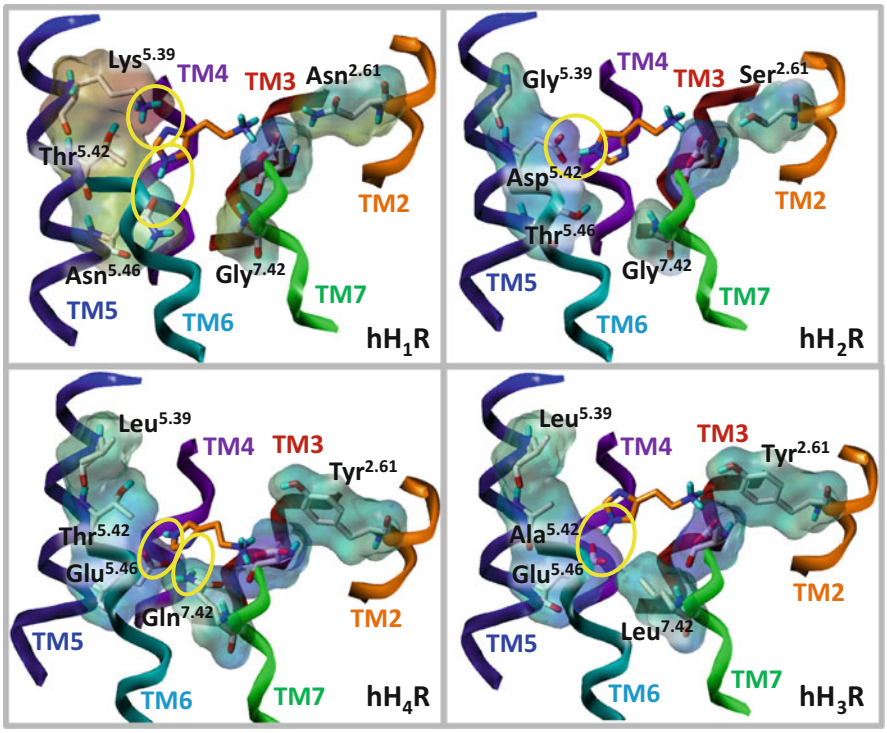


Fig. 7 Comparison of the binding mode of histamine, docked into the four human histamine receptor subtypes (*yellow circles*: most important interactions between the respective receptor and histamine)

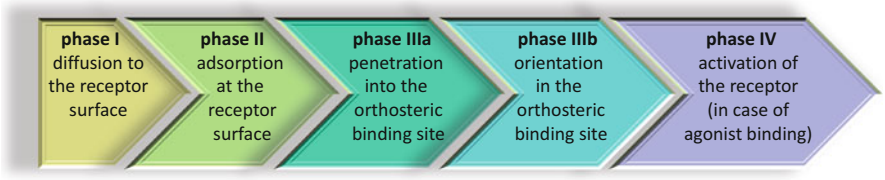


Fig. 8 Different phases of the whole binding process of a ligand into its binding site of a receptor

observed to interact with the histamine. In the orthosteric binding pocket, the positively charged amine moiety of the histamine established a stable interaction with $Gln^{7.42}$ and the highly conserved $Asp^{3.32}$. Furthermore, the NH of the imidazole moiety formed a stable hydrogen bond with $Glu^{5.46}$. This observation is in good accordance to mutagenesis studies, because for the $Glu^{5.46}$Gln mutant, the affinity of the histamine to the hH_4R decreased significantly (Jongejan et al. 2008).

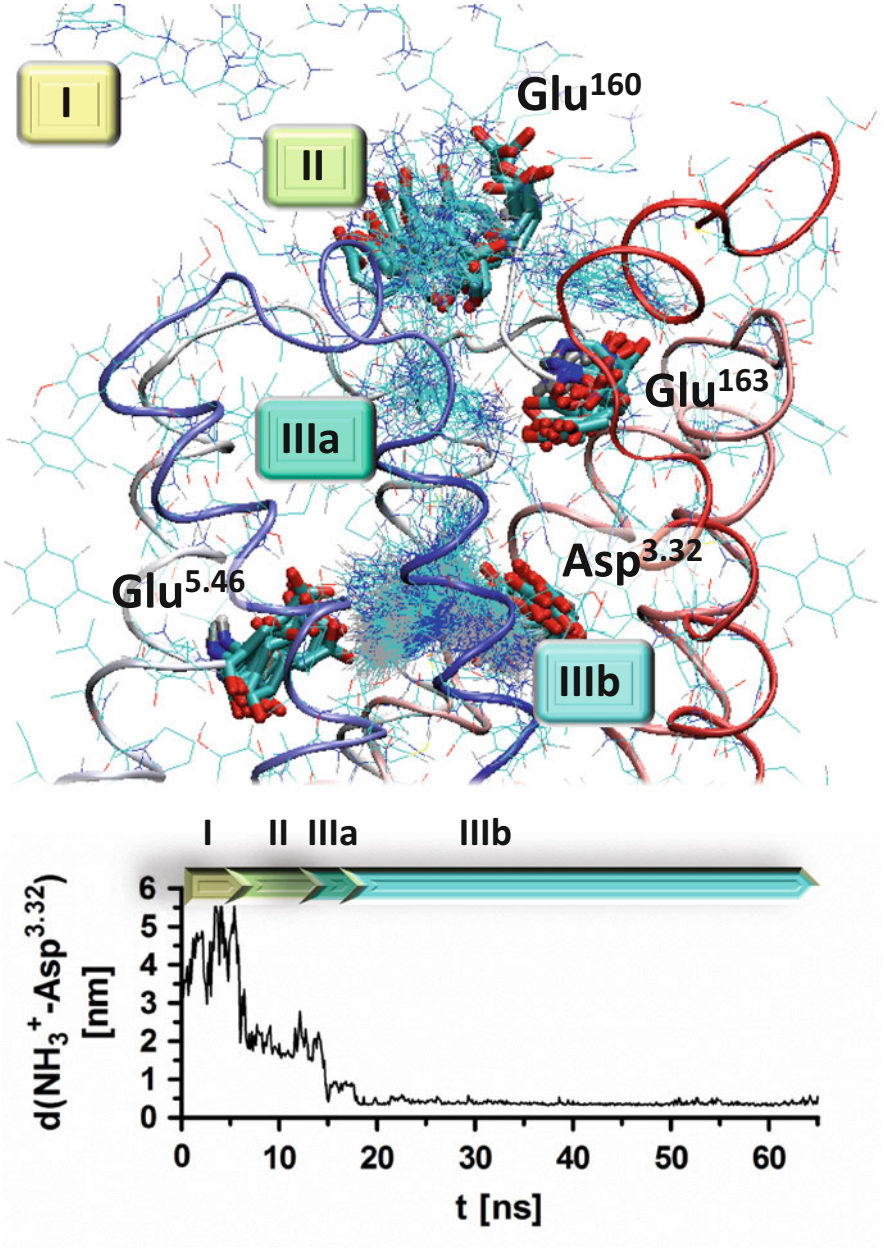


Fig. 9 Binding pathway of histamine from the extracellular side into the orthosteric binding site of the hH_4R by unconstrained MD simulations [modified according to Wittmann and Strasser (2015)]

One first advantage of such MD simulations is that the ligand "finds" its binding mode without any constraints. In contrast, if the ligand is docked into the binding pocket and a subsequent MD simulation is performed, the binding mode of the ligand is possibly biased by the investigator. Of course, it has to be mentioned that such calculations are in general very time consuming and are only described for the β_2R (Dror et al. 2011), hH_4R (Wittmann and Strasser 2015), D_2R and D_3R (Thomas et al. 2016) until now. A second advantage of such MD simulations is that the amino acids, being involved in the ligand binding, can be identified, which is not possible by crystal structures, because here, the ligand is already bound into the binding pocket. Although the binding pathway has to be supported by mutagenesis and pharmacological studies, MD simulation so far is the only technique, which allows to observe the dynamic behaviour of ligand and receptor on a molecular level. And thus, MD simulation is an important and powerful technique to increase the understanding of histamine receptors on a molecular level. However, MD simulations are very time consuming, and only some hundred μs can be simulated until now, which may not be enough to observe the whole agonist binding and the related receptor-activation process.

4.3 Different Orientations of Ligands in the Binding Pocket

Phenylhistamines and histaprodifens (Fig. 10), H_1R partial agonists, were developed as tools to study different histamine H_1 receptor species in intact cell systems and in the Sf9 expression system (Leschke et al. 1995; Malinowska et al. 1999; Elz et al. 2000; Menghin et al. 2003; Seifert et al. 2003; Strasser et al. 2008a, 2009).

Pharmacological studies showed that histaprodifen and suprahistaprodifen show higher affinity to gpH_1R than to hH_1R (Strasser et al. 2008a, 2009). It is important to

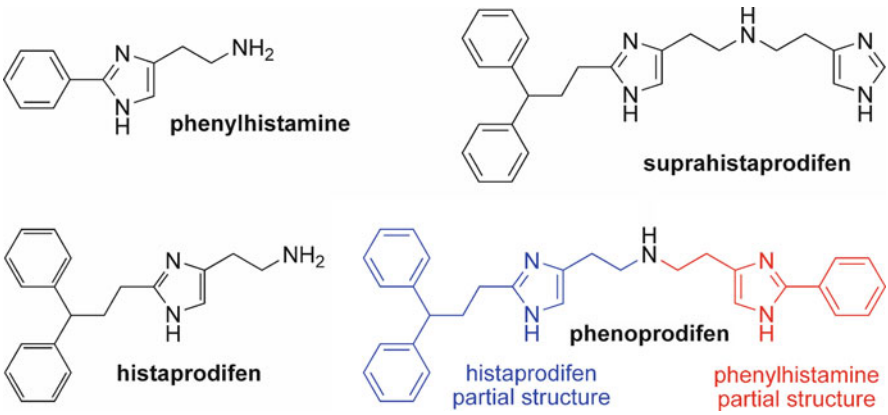


Fig. 10 Structures of the H_1R partial agonists phenylhistamine (Strasser et al. 2009), histaprodifen (Strasser et al. 2008a), suprahistaprodifen (Strasser et al. 2008a) and phenoprodifen (Strasser et al. 2008a)

analyse species differences on a molecular level by combined mutagenesis and molecular modelling studies: Those studies increase the understanding of histamine receptors on a molecular level, e.g. with regard to subtype or species selectivity, which is important to develop new and more efficient drugs for therapy. By mutagenesis studies, the amino acid at position 2.61 was identified to act as a selectivity switch for suprahistaprodifen between gpH_1R (Ser) and hH_1R (Asn), but not for histaprodifen (Bruysters et al. 2005). Molecular modelling studies and MD simulations suggest that the smaller histaprodifen is bound into the main pocket (I, Fig. 5) near to TM5 and is, in contrast to the more bulky suprahistaprodifen, not in contact with TM2 of pocket II (Fig. 5) (Bruysters et al. 2005; Strasser et al. 2008a). Furthermore, the amino acid at position 2.61 may be involved in subtype or species differences at several histamine receptors for bulky ligands, which also occupy the second part of the orthosteric binding pocket (II, Fig. 5).

From a modelling point of view, an interesting class of partial agonists at the H_1R are the phenoprodifens, hybrid compounds, comprising a histaprodifen and phenylhistamine partial structure (Fig. 10) (Strasser et al. 2008a). Since histaprodifens and phenylhistamines were suggested to bind in a pocket between TM3, TM5 and TM6, phenoprodifens were assumed to be able to bind in two different orientations into the orthosteric binding site of H_1R (Bruysters et al. 2004; Strasser et al. 2009; Strasser and Wittmann 2010). The MD simulations showed differences in ligand-receptor interaction energy for phenoprodifen (Strasser et al. 2009): At hH_1R, orientation 1 (diphenylpropylmoiety near to TM5) is preferred compared to orientation 2 (diphenylpropylmoiety near to TM2), while at gpH_1R, none of both orientations is preferred. Furthermore, it is supported by QSAR studies that the orientation of phenoprodifens and suprahistaprodifens is dependent on the ligand structure and the H_1R species (Strasser and Wittmann 2010). Although it is very hard to verify two different binding orientations of a ligand by experimental studies, e.g. by X-ray crystallography, considering two different binding orientations of ligands may be an important approach in development of new ligands, especially with regard to heterobivalent ligands.

4.4 Scaffold Hopping Approach to Identify New Ligand Classes

Experimental and virtual high-throughput screening is an established, but more or less time- and cost-consuming method to identify new ligands for a distinct target (Kumari et al. 2015). By contrast, based on a scaffold hopping approach starting from the quinoxalines (Smits et al. 2008b), new quinazolines were identified as highly potent H_4R inverse agonists (Smits et al. 2008a): A side pocket with hydrophobic properties within the orthosteric binding site of the H_4R was proposed by a fragment-based approach (Fig. 11) (Smits et al. 2008a): Based on these findings it was suggested that the same pocket could be occupied by substituents in 2-position of the quinoxaline and 4-position of the quinazoline moiety. Furthermore, based on a structural comparison of the quinazoline and quinoxaline scaffold, it is suggested that both moieties are similar regarding their binding mode in the

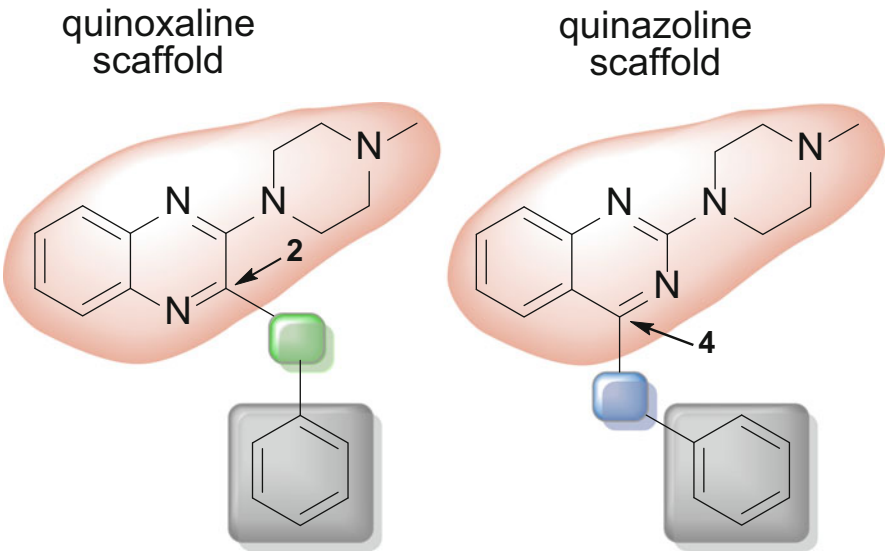


Fig. 11 Scaffold hopping approach to develop a new class of hH_4R ligands [modified according to Smits et al. (2008a)]

orthosteric binding site of the hH_4R. This study is a nice example that a scaffold hopping approach may be a useful approach to identify new classes of ligands not only at the H_4R, but also at the other histamine receptor subtypes.

4.5 Impact of Molecular Modelling Studies to Explain the Pharmacology of Phenylhistamines at the hH_4R

Phenylhistamines were identified as partial agonists at the H_1 receptor (Strasser et al. 2009). But recent pharmacological studies showed that *N*-methylated and/or CF_3- or Br-substituted phenylhistamines show a higher affinity to the hH_4R than to the hH_1R (Wittmann et al. 2011). The exchange of $R^1 = H \rightarrow R^1 = CH_3$ and $R^2 = H \rightarrow R^2 = Br,CF_3$ leads to an increase in affinity of two orders of magnitude at the hH_4R (Fig. 12) (Wittmann et al. 2011). Subsequent MD simulations of the phenylhistamines showed that the methyl group (R^1) and/or the Br/CF_3 (R^2) bind into two small subpockets 1 (R^1) and 2 (R^2) of the hH_4R, which are not occupied by the unsubstituted phenylhistamine. Furthermore, the predicted Gibbs energies for the transfer of the ligand from the aqueous phase into the orthosteric binding pocket are in very good correlation with the experimentally determined affinities. This is a good example to demonstrate that molecular modelling studies are able to explain pharmacological data on a molecular level. However, it has to be taken into account that the ligands investigated within this study are structurally highly related and the predictive possibilities of molecular modelling studies might decrease in case of compounds with large structural differences.

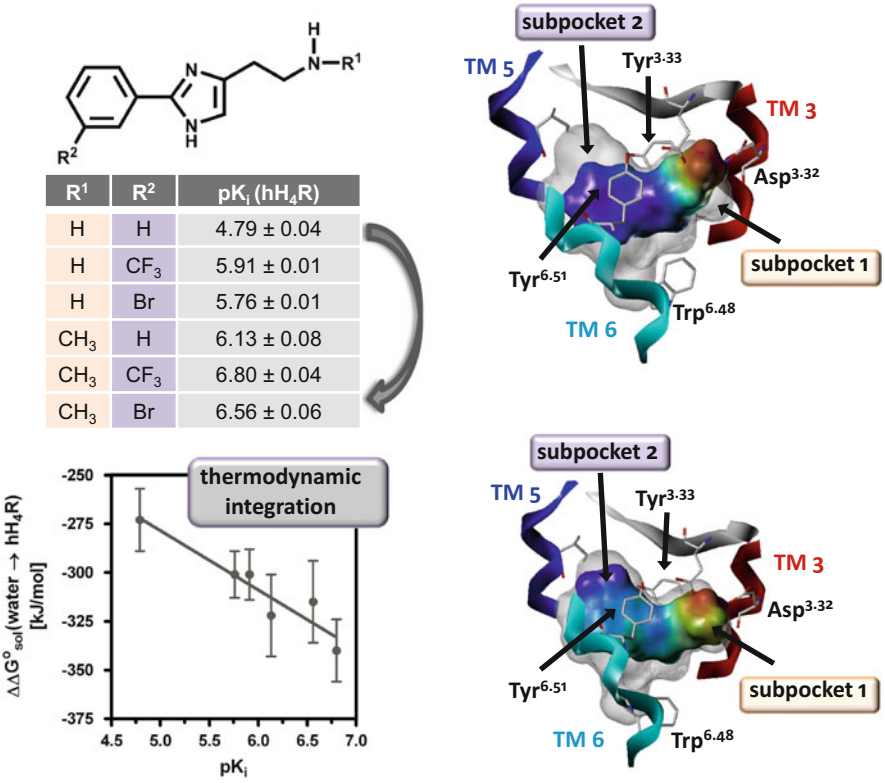


Fig. 12 Influence of small substituents in phenylhistamine onto affinity at the hH_4R—a structural and energetical analysis [modified according to Wittmann et al. (2011)]

4.6 Influence of Monovalent Cations and Anions to the Histamine H_3 and H_4 Receptor

It was shown by experimental studies that the concentration of sodium ions has influence on the pharmacological data, e.g. potency or basal activity of the receptor at the hH_3R or hH_4R (Schneider et al. 2009; Schnell and Seifert 2010): With increasing concentration of NaCl, a decreasing basal activity of the hH_3R and hH_4R was observed, indicating that the inactive conformation of the receptor is stabilised. Based on experimental studies at several GPCRs (Selent et al. 2010; Katritch et al. 2014; Strasser et al. 2015), it was supported by MD simulations that Na^+ is able to bind to the allosteric binding site near $Asp^{2.50}$ at hH_3R and hH_4R (Fig. 13a–c) (Wittmann et al. 2014b). Recently, MD simulations were used to study the binding pathway of a sodium ion from the extracellular side via the orthosteric binding site into the allosteric binding site at the hH_4R (Wittmann et al. 2014b; Strasser et al. 2015). The analysis of the ion entry path into the receptor showed that it is quite the same as for histamine at the hH_4R (see Fig. 9) (Wittmann et al. 2014b; Strasser et al. 2015). Furthermore, the MD simulations suggest that the presence or

absence of a sodium ion in the allosteric binding site may have influence on the binding mode of ligands, e.g. thioperamide at the hH_3R (Wittmann et al. 2014a), which may explain differences in potencies in dependence of the NaCl concentration. In the MD simulations of a Na^+ in its allosteric binding site near to $Asp^{2.50}$ at hH_3R and hH_4R a water chain, connecting the highly conserved $Asp^{3.32}$ of the orthosteric and $Asp^{2.50}$ of the allosteric binding site, was observed (Wittmann et al. 2014b): While this water chain is continuous at the hH_3R, it is disrupted, but bridged by $Gln^{7.42}$ at hH_4R. So far, it remains unclear if this water chain plays a role in receptor activation or subtype differences between hH_3R and hH_4R. A systematic analysis of the influence of monovalent cations (Li^+, Na^+, K^+) and anions (Cl^-, Br^-, I^-) on the hH_3R showed that not only cations but also anions have an influence on the hH_3R (Schnell and Seifert 2010), which is dependent on the chemical nature of the analysed monovalent ion. The MD simulations suggest that the depth of binding of the monovalent cation depends on its size (Fig. 13c), which may explain the different influence of cations on pharmacology of GPCRs (Schnell and Seifert 2010; Strasser et al. 2015). Furthermore, it is suggested that small positively charged ligands may be able to bind into the highly conserved Na^+ pocket near to $Asp^{2.50}$, e.g. the diuretic drug amiloride to the adenosine A_{2A} receptor (Katritch et al. 2014). A similar observation was made during MD simulations of thioperamide in the binding pocket of the hH_3R: In the presence of a sodium ion in the allosteric binding site, the thioperamide remains quite stable in the orthosteric binding pocket, but in the absence of a Na^+ in the allosteric binding site, the positively charged imidazole moiety of the thioperamide moved between the orthosteric $Asp^{3.32}$ and the allosteric $Asp^{2.50}$ (Wittmann et al. 2014a). Thus, it is suggested that small molecules, able to bind in the allosteric Na^+-binding site, may exhibit new functional properties or may open new opportunities in therapy (Katritch et al. 2014).

It was shown by MD simulations with monovalent cations and anions in the aqueous phase that the monovalent anions preferably bind between the intracellular part of the receptor, because in this region, some positively charged amino acids are located (Strasser et al. 2015). Since this is the same region for binding of the Gα-subunit, it is suggested that monovalent anions are involved in regulation of the interaction between receptor and Gα-subunit.

5 Conclusions and Future Studies

A large number of studies combining experimental (synthesis, pharmacological experiments, mutagenesis) and modelling techniques (QSAR, docking, MD simulation) addressing the histamine receptors were performed, reflecting that only a combination of several experimental and modelling techniques leads to an increased understanding of the histamine receptors on molecular level (Fig. 1) and provides synergistic input to each other. Although molecular modelling techniques are a powerful tool to obtain more detailed insights into histamine receptors (Table 5), it is necessary to proof or support the modelling results with experimental studies. However, one great advantage of modelling studies is that they allow to obtain deeper

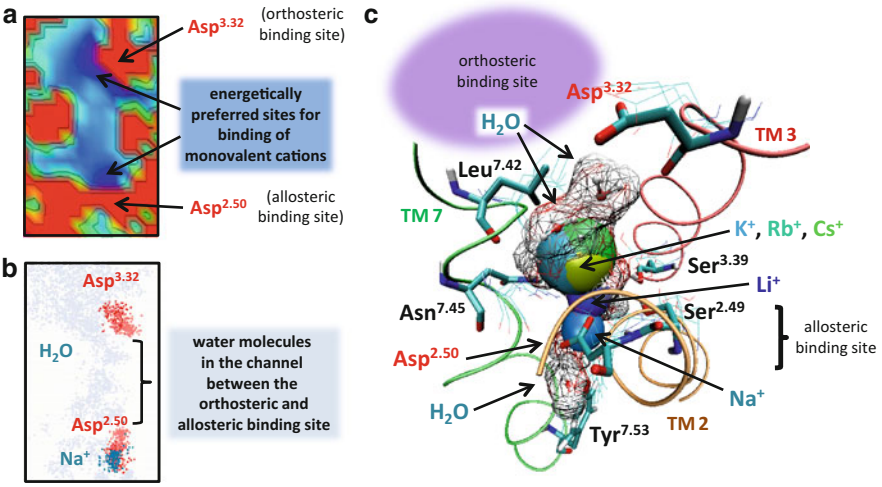


Fig. 13 The allosteric cation-binding site near $Asp^{2.50}$. (**a**) Interaction energy surface of Na^+ with the orthosteric and allosteric binding site of the hH_3R (*blue*: energetically preferred regions for the Na^+). (**b**) Preferred areas for the Na^+ in the allosteric binding site and water in the water channel and the orthosteric and allosteric binding site. (**c**) Overlay of the most preferred position of Li^+, Na^+, K^+, Rb^+ or Cs^+ in the allosteric binding site of the hH_3R according to MD simulations [modified according to Strasser et al. (2015)]

Table 5 Gain of knowledge in histamine receptor research by molecular modelling

Gain of knowledge	Histamine receptor	Most important modelling techniques
– Binding mode of different ligands in the orthosteric binding site – Amino acids, involved in ligand binding – Detection of different ligand binding orientations	xH_xR, mutated xH_xRs	Docking, molecular dynamics, ab initio calculations
– Binding pathways of histamine into the orthosteric binding pocket – Amino acids, involved in ligand recognition	hH_4R	Molecular dynamics
– Binding pathway of Na^+ into the allosteric binding pocket	hH_3R, hH_4R	Molecular dynamics
– Preferred binding sites of monovalent cations and anions – Modelling of a GPCR–G protein complex	hH_4R	Molecular dynamics
– Prediction of affinities	xH_xR	QSAR

insights into the histamine receptors on a molecular level that will be complementary and even synergistic to experimental techniques.

Until now, a large number of questions in the histamine research were solved amongst others by molecular modelling studies (Table 5). However, there is a large

number of remaining questions: For example, there is only little knowledge about the interactions between histamine receptors and G proteins or β-arrestin or about heterodimers on a molecular level. In future, modelling studies should focus on those questions, because they can provide important hints for mutagenesis studies to decode the interaction between a receptor and a specific G protein or for development of biased or bivalent ligands.

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Pharmacological Characterization of Human Histamine Receptors and Histamine Receptor Mutants in the Sf9 Cell Expression System

Erich H. Schneider and Roland Seifert

Contents

Abstract

A large problem of histamine receptor research is data heterogeneity. Various experimental approaches, the complex signaling pathways of mammalian cells, and the use of different species orthologues render it difficult to compare and interpret the published results. Thus, the four human histamine receptor subtypes were analyzed side-by-side in the Sf9 insect cell expression system, using radioligand binding assays as well as functional readouts proximal to the receptor activation event (steady-state GTPase assays and [^{35}S]GTPγS assays). The human H_1R was co-expressed with the regulators of G protein signaling RGS4 or GAIP, which unmasked a productive interaction between hH_1R and insect cell $G\alpha_q$. By contrast, functional expression of the hH_2R required the generation of

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an hH_2R-Gsα fusion protein to ensure close proximity of G protein and receptor. Fusion of hH_2R to the long ($Gs\alpha_L$) or short ($Gs\alpha_S$) splice variant of $G\alpha_s$ resulted in comparable constitutive hH_2R activity, although both G protein variants show different GDP affinities. Medicinal chemistry studies revealed profound species differences between hH_1R/hH_2R and their guinea pig orthologues gpH_1R/gpH_2R. The causes for these differences were analyzed by molecular modeling in combination with mutational studies. Co-expression of the hH_3R with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{i/o}$ in Sf9 cells revealed high constitutive activity and comparable interaction efficiency with all G protein isoforms. A comparison of various cations (Li^+, Na^+, K^+) and anions (Cl^-, Br^-, I^-) revealed that anions with large radii most efficiently stabilize the inactive hH_3R state. Potential sodium binding sites in the hH_3R protein were analyzed by expressing specific hH_3R mutants in Sf9 cells. In contrast to the hH_3R, the hH_4R preferentially couples to co-expressed $G\alpha_{i2}$ in Sf9 cells. Its high constitutive activity is resistant to NaCl or GTPγS. The hH_4R shows structural instability and adopts a G protein-independent high-affinity state. A detailed characterization of affinity and activity of a series of hH_4R antagonists/inverse agonists allowed first conclusions about structure/activity relationships for inverse agonists at hH_4R. In summary, the Sf9 cell system permitted a successful side-by-side comparison of all four human histamine receptor subtypes. This chapter summarizes the results of pharmacological as well as medicinal chemistry/molecular modeling approaches and demonstrates that these data are not only important for a deeper understanding of H_xR pharmacology, but also have significant implications for the molecular pharmacology of GPCRs in general.

Keywords

[^{35}S]GTPγS binding • GPCRs • Histamine receptors • Radioligand binding • Sf9 insect cells • Steady-state GTPase assay

Abbreviations

[^{3}H]histamine	Tritiated histamine
[^{3}H]NAMH	Tritiated N^{α}-methylhistamine
[^{35}S]GTPγS	GTPγS, labeled with ^{35}S
α_2AR	α-Adrenoceptor, subtype 2
β_1AR, β_2AR	β-Adrenoceptor subtypes
β_2AR_{CAM}	β_2-Adrenoceptor, constitutively active mutant
[γ-^{32}P]GTP	GTP, γ-labeled with ^{32}P
$A_{2a}R$	Adenosine receptor subtype 2A
AC	Adenylyl cyclase
ACKR1	Atypical chemokine receptor 1

AIPGs	N^G-acylated imidazolylpropylguanidines
B_2R	Bradykinin B_2 receptor
Balb/C, C57Bl/6	Mouse strains
cAMP	3′,5′-Cyclic adenosine monophosphate
CCR5	C–C chemokine receptor type 5
CNS	Central nervous system
D_1R, D_2R	Dopamine receptor subtypes
DRY	Aspartate–arginine–tyrosine motif at the bottom of the third transmembrane helix of a GPCR
e	Extracellular loop (e.g. e2)
EAE	Experimental autoimmune encephalitis
ECL	Extracellular loop
FLAG	Peptide tag (DYKDDDDK)
FPR1	Formyl peptide receptor 1
FPR26	FPR1 isoform
$GABA_BR$	Receptor for γ-amino butyric acid, subtype B
GAIP	Gα-interacting protein (= regulator of G protein signaling RGS19)
GDP	Guanosine-5′-diphosphate
gp	Guinea pig (prefix)
GPCR	G protein-coupled receptor
$Gs\alpha_L$	Stimulatory G protein, long splice variant
$Gs\alpha_S$	Stimulatory G protein, short splice variant
GTP	Guanosine-5′-triphosphate
GTPγS	Guanosine 5′-*O*-[γ-thio]triphosphate (non-hydrolysable GTP derivative)
$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{i/o}$	Inhibitory G protein isoforms
$G\alpha_q$	G protein isoform activating phospholipase C
$G\alpha_s$	Stimulatory G protein
$G\beta_1\gamma_2$	G protein complex, consisting of $G\beta_1$ and $G\gamma_2$
h	Human (prefix)
h(gpE2)H_1R	Chimeric receptor (human H_1R with second extracellular loop from guinea pig H_1R)
h(gpNgpE2)H_1R	Chimeric receptor (human H_1R with N-terminus and second extracellular loop from guinea pig H_1R)
H_1R, H_2R, H_3R, H_4R	Histamine receptor subtypes
HDC	Histidine decarboxylase
HeLa	Cervix carcinoma cell line
His_6	Hexahistidine tag
HL-60	Human promyelocytic leukemia cell line
HPLC-MS/MS	High performance liquid chromatography-coupled tandem mass spectrometry
K_D	Ligand dissociation constant
K_M	Michaelis–Menten constant, substrate concentration resulting in 50% of maximum enzymatic reaction speed
LH/CG receptor	Receptor for luteinizing hormone/choriogonadotropin

m	Murine (prefix)
M_3R	Muscarinic receptor subtype 3
NAMH	N^{α}-methylhistamine
NgpChH_2R-Gsα_S	Fusion protein of Gsα_S with a chimeric receptor (N-terminus to transmembrane domain 3 from guinea pig H_2R plus transmembrane domain 4 to C-terminus from human H_2R)
NhCgpH_2R-Gsα_S	Fusion protein of Gsα_S with a chimeric receptor (N-terminus to transmembrane domain 3 from human H_2R plus transmembrane domain 4 to C-terminus from guinea pig H_2R)
pEC_{50}	Negative decadic logarithm of the agonist concentration that causes 50% of the maximum effect
pIC_{50}	Negative decadic logarithm of the antagonist concentration that causes 50% inhibition
pK_b	Negative decadic logarithm of a dissociation constant determined in a functional assay
PKC	Protein kinase C
pK_i	Negative decadic logarithm of a dissociation constant determined in a competition binding assay
PLC	Phospholipase C
PTX	Pertussis toxin
r	Rat (prefix)
RAMH	(R)-α-methylhistamine
RGS4	Regulator of G protein signaling 4
SAR	Structure-activity relationship
S49	Murine lymphoma cell line
Sf9, Sf21	Insect cell lines originating from ovarian cells of *Spodoptera frugiperda*
Th1, Th2	Differentially polarized T helper cell subgroups
TM	Transmembrane helix of a G protein-coupled receptor
TMN	Tuberomamillary nucleus
U373 MG	Human astrocytoma cell line
V_{max}	Maximum enzymatic reaction speed in the presence of saturating substrate concentrations

1 Principles of GPCR Analysis in the Sf9 Cell Expression System

1.1 The Sf9 Cell Expression System

Pharmacological characterization of GPCRs is commonly performed in transfected mammalian cells or in cells that endogenously express the receptor of interest (Kenakin 1996). There are, however, several problems of mammalian cell systems.

First, mammalian cells normally express various additional GPCRs, which may result in GPCR heteromerization or signaling crosstalk (Breitwieser 2004; Prezeau et al. 2010; Gomes et al. 2016). For example, signaling crosstalk between GPCRs has been described for the $G\alpha_i$-coupled $GABA_BR$ and the $G\alpha_q$-coupled $mGlu_{1A}R$ (Rives et al. 2009). Another example is ACKR1 (atypical chemokine receptor 1), which has been shown to functionally antagonize CCR5 by forming ACKR1/CCR5 heterodimers (Chakera et al. 2008). Second, the presence of other constitutively active receptors may interfere with the analysis of agonist-independent activity of the receptor of interest. For example, the inverse FPR1 agonist cyclosporin H failed to inhibit basal $G\alpha_i$ protein activity in HL-60 cells, indicating that these cells additionally express other constitutively active receptors different from FPR1 (Wenzel-Seifert and Seifert 1993; Seifert and Wenzel-Seifert 2003). Third, promiscuous G protein coupling of GPCRs in the presence of several G protein subtypes may preclude the analysis of GPCR-G protein selectivity (Woehler and Ponimaskin 2009). Finally, some GPCRs are only expressed at low levels in mammalian cells, rendering it difficult to obtain a sufficiently high signal-to-noise ratio in functional and ligand binding assays.

As discussed in a comprehensive review article (Schneider and Seifert 2010c), the problems listed above are effectively addressed by using the Sf9 cell expression system. Sf9 cells are derived from the Sf21 cell line, which had been originally isolated from the pupal ovarian tissue of the American fall army worm (*Spodoptera frugiperda*). The protein of interest is expressed by infecting Sf9 cells with baculoviruses encoding the corresponding gene. Although Sf9 cells express $G\alpha_i$-, $G\alpha_q$-, and $G\alpha_s$-like proteins, insect cell $G\alpha_i$ is not activated by mammalian GPCRs. This renders Sf9 cells a functionally "$G\alpha_i$-free" system and permits the analysis of $G\alpha_i$-coupled receptors without the necessity of pertussis toxin (PTX)-mediated GPCR/$G\alpha_i$ uncoupling. Also, PTX would not be active in Sf9 cells, because it does not enter the cells (Wenzel-Seifert et al. 1998). By contrast, uncoupling of $G\alpha_i$-coupled GPCRs by PTX in mammalian cells is problematic. Despite entering mammalian cells, PTX is not capable of completely inactivating all $G\alpha_i$ proteins (Wenzel-Seifert and Seifert 1990).

Moreover, Sf9 cells do not express constitutively active GPCRs and therefore provide a low-background environment for the analysis of agonist-independent receptor activity. Furthermore, the highly efficient baculovirus promoters lead to very high expression levels of GPCRs in Sf9 cells. This results in high signal-to-noise ratios in binding assays and allows the purification of receptor protein, e.g. for crystallization purposes. Finally, as explained below, Sf9 cell membranes expressing large amounts of GPCRs and G proteins can be used to study G protein activation in steady-state GTPase assays and experiments with [^{35}S]GTPγS ([^{35}S]-labeled guanosine 5′-*O*-[γ-thio]triphosphate).

For the preparation of baculoviruses encoding the gene of interest, several straightforward methods are established. The Sf9 cell studies discussed in this chapter were performed by using the BaculoGold™ kit from Invitrogen. As explained in Fig. 1, the gene of interest (in this example hH_4R) is cloned into a pVL1392 baculovirus transfer vector, which is transfected into Sf9 cells together with the missing part of the baculovirus genome (BaculoGold™ DNA).

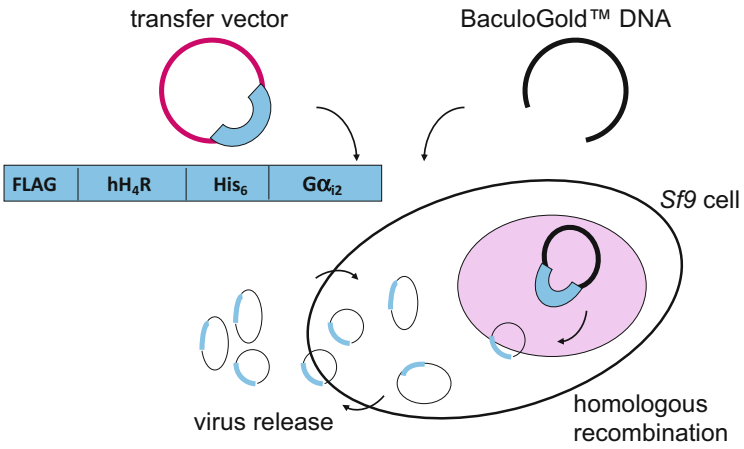

Fig. 1 Preparation of baculoviruses for the expression of a GPCR (example for H_4R-$G\alpha_{i2}$ fusion protein). The gene of interest (in this case a FLAG-tagged hH_4R fused to $G\alpha_{i2}$ via a His_6 linker) is cloned into a pVl1392 transfer vector. The plasmid and the missing part of the baculovirus DNA (BaculoGold™ DNA) are co-transfected into Sf9 cells. The full baculovirus genome is completed in Sf9 cells by homologous recombination. The cells start to release virus particles into the surrounding medium

After that, the full baculovirus genome with the integrated receptor gene is reconstituted in the host cells by homologous recombination. The cell then releases virus particles into the surrounding medium which is harvested and used for further infections. A detailed protocol for the production and maintenance of genetically modified baculoviruses was published in *Methods in Enzymology* (Schneider and Seifert 2010a). Numerous examples of the characterization of $G\alpha_q$-, $G\alpha_s$-, and $G\alpha_i$-coupled receptors reconstituted in Sf9 insect cells were documented by Schneider and Seifert (2010c). In this chapter, an in-depth discussion of the pharmacological characterization of histamine receptors in Sf9 cell membranes is provided.

1.2 Methods for the Characterization of Histamine Receptors in Sf9 Cell Membranes

1.2.1 The G Protein Cycle

The G protein activation cycle (Gilman 1987; Oldham and Hamm 2008), which is explained in the following, is the basis for the methods used to generate the functional histamine receptor data discussed in this chapter. When histamine binds to the hH_4R, the receptor protein undergoes a conformational change and interacts with an inactive GDP-bound heterotrimeric G protein (Fig. 2 step 1). This induces GDP release and the formation of the so-called ternary complex, which contains agonist, receptor and guanine-nucleotide-free G protein (Fig. 2, step 2). It is generally accepted that a GPCR exhibits its highest agonist-binding affinity, when it is part of the ternary complex. The interaction between agonist-bound

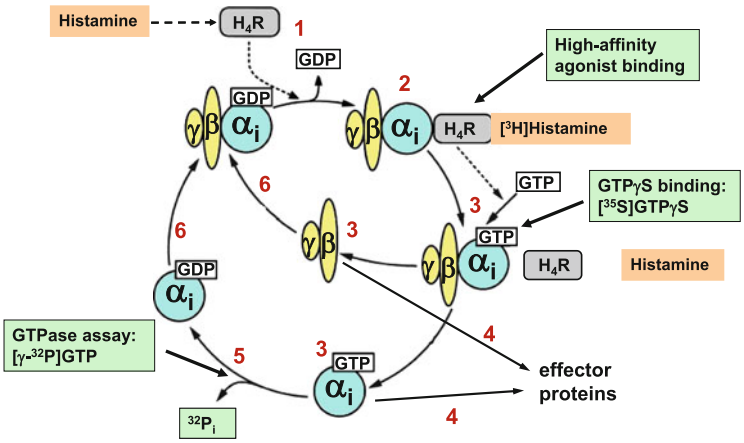

Fig. 2 Stimulation of $G\alpha_i$-proteins by the histamine H_4R and resulting G protein cycle. The numbers designate the different stages of the cycle and are explained in detail in Sect. 1.2.1

GPCR and G protein promotes GTP binding to the Gα-subunit. This weakens the intermolecular interactions in the G protein and in the ternary complex, breaking the complex up into agonist and GPCR as well as Gα- and Gβγ subunit (Fig. 2, step 3).

After their dissociation from the receptor, the active GTP-loaded Gα subunit and the Gβγ part interact with various effector proteins (Fig. 2, step 4) and induce numerous biochemical processes. Such effects include activation ($G\alpha_s$) or inhibition ($G\alpha_i$) of membranous adenylyl cyclase (AC), modulation of ion channel activity (Gβγ, $G\alpha_i$) or stimulation of phospholipase C (PLC) activity followed by intracellular Ca^{2+} mobilization (Gβγ, $G\alpha_q$). As long as GTP is bound to Gα, the Gα and Gβγ subunits are active. To terminate signaling, the Gα subunit inactivates itself by its intrinsic GTPase activity, resulting in conversion of the bound GTP to GDP and release of inorganic phosphate (Fig. 2, step 5). The inactive GDP-bound Gα subunit re-associates with Gβγ and becomes available for another cycle (Fig. 2, step 6).

1.2.2 High Affinity Radioligand Binding

High affinity radioligand binding with histamine receptors is performed with radiolabeled agonists, e.g. tritiated histamine ([^{3}H]histamine). Normally, agonists show their highest affinity to the ternary complex (Fig. 2, step 2) and stabilize the active receptor conformation. Thus, agonistic radioligands preferentially label the G protein-coupled high-affinity receptor population. When two populations of GPCRs with different G protein coupling states occur simultaneously, the saturation or competition curves with agonistic radioligands may become biphasic, which allows the determination of high-affinity and a low-affinity binding constants. This was, e.g., demonstrated for histamine H_2R (Houston et al. 2002) as well as for the β_2-adrenergic receptor (β_2AR) or the dopamine D_1R (Gille and Seifert 2003). By contrast, inverse agonists interact preferentially with the inactive receptor state and

therefore show increased affinity to uncoupled GPCRs. Neutral antagonists do not discriminate between active and inactive receptor states and label both receptor conformations with comparable affinity.

For some experiments it may be required to convert GPCRs to their inactive conformation by disrupting receptor-G protein interactions. This is achieved by addition of GTPγS (guanosine 5′-*O*-[γ-thio]triphosphate), which binds to the G-α-subunit like GTP (Fig. 2, step 3), but cannot be hydrolyzed by the Gα subunit (Gilman 1987). Thus, no GDP-loaded G protein is available anymore for the formation of new ternary complexes resulting in uncoupling of the entire GPCR population. This is normally reflected by a dramatic reduction in the binding affinity of agonistic radioligands. A detailed protocol for high-affinity agonist binding assays as well as example data for various receptor/G protein systems is provided in book chapters about GPCR/G protein co-expression and fusion protein systems in Sf9 cell membranes (Schneider and Seifert 2010a, b).

1.2.3 Steady-State GTPase Assays

In steady-state GTPase assays, the intrinsic GTPase activity (Fig. 2, step 5) of the active GTP-bound Gα-subunit is determined (Gilman 1987; Schneider and Seifert 2010a). This is achieved by quantitating radioactive inorganic phosphate released after Gα-mediated hydrolysis of [γ-^{32}P]GTP. The steady-state GTPase assay represents a very proximal readout of GPCR activation, which directly reflects GPCR-mediated G protein stimulation. By contrast, functional assays analyzing more distal parameters (e.g., Ca^{2+}-, cAMP- or reporter gene assays) are often influenced by signal amplification processes, making valid conclusions about the original extent of receptor activation difficult. Technical details of the steady-state GTPase assay were explained in two book chapters about GPCR/G protein co-expression and fusion protein systems in Sf9 cells (Schneider and Seifert 2010a, b).

Steady-state GTPase assays can be used for the functional characterization of ligands in medicinal chemistry projects. In addition, these assays provide information about the efficacy of receptor-G protein interactions. In Michaelis–Menten kinetics experiments with increasing concentrations of the substrate [γ-^{32}P]GTP, the K_M and V_{max} value of the Gα-GTPase can be determined (Schneider and Seifert 2009, 2010a). Subtraction of the GTPase activity in the presence of a full inverse agonist from the activity elicited by a full agonist yields the total receptor-regulated GTPase activity (ΔV_{max}). Dividing the ΔV_{max} value by B_{max} (maximum number of radiolabeled receptor proteins) provides the so-called turnover number, which signifies the number of GTP molecules hydrolyzed per minute, resulting from the activation of a single GPCR protein (Schneider and Seifert 2010a).

1.2.4 [^{35}S]GTPγS Binding Assays

The [^{35}S]GTPγS binding assay is another method to determine the functional effect of a ligand at a very proximal level of GPCR signal transduction. As depicted in Fig. 2, GTPγS binds to the activated Gα-subunit instead of GTP, resulting in the dissociation of the ternary complex (Fig. 2, step 3). Unlike GTP, however, GTPγS

cannot be hydrolyzed by the intrinsic GTPase activity of Gα (Gilman 1987), resulting in an accumulation of GTPγS-bound Gα-subunits. When radiolabeled [^{35}S]GTPγS is used, the amount of activated Gα subunits can be quantitated by scintillation counting, allowing the characterization of Gα activation kinetics (time course of [^{35}S]GTPγS-Gα accumulation) and the determination of agonist- and inverse-agonist modulated Gα activation. When the total ligand-regulated Gα activation (maximum effect of full agonist minus activation level in the presence of a full inverse agonist) is divided by the B_{max} value from radioligand binding, the so-called coupling factor is obtained. Similar to the aforementioned turnover number, the coupling factor provides information about the number of Gα subunits stimulated by a single GPCR protein.

Furthermore, saturation binding experiments with increasing concentrations of [^{35}S]GTPγS yield information about alterations of Gα affinity to [^{35}S]GTPγS under various conditions (e.g., constitutive receptor activity, agonist- or inverse agonist-induced effects). Finally, [^{35}S]GTPγS binding assays are useful to pharmacologically characterize new ligands synthesized during the course of medicinal chemistry projects. A detailed experimental protocol of [^{35}S]GTPγS binding assays as well as an explanation of how to analyze and interpret the data is provided in comprehensive book chapters about the characterization of GPCR/Gα co-expression and fusion protein systems in Sf9 cell membranes (Schneider and Seifert 2010a, b).

1.2.5 Fusion Protein Systems

Mammalian GPCRs and G protein Gα and Gβγ subunits can be readily co-expressed in the baculovirus/Sf9 cell system yielding useful systems for the pharmacological characterization of GPCR ligands and receptor-G protein interactions. However, sometimes co-expression systems produce only insufficient GPCR-mediated Gα activation (Seifert et al. 1998a; Gille and Seifert 2003). Specifically, $G\alpha_s$ proteins rapidly dissociate from the plasma membrane (Yu and Rasenick 2002) and therefore cannot be efficiently activated by a co-expressed GPCR. This problem is solved by constructing GPCR-Gα fusion proteins (Fig. 3) that guarantee close proximity of receptor and G protein.

This approach was successfully used for the pharmacological characterization of $G\alpha_s$-coupled receptors like the β_2AR (Bertin et al. 1994; Seifert et al. 1998a) or the histamine H_2R (Wenzel-Seifert et al. 2001). GPCR-Gα fusion proteins of β_2AR, FPR1 or dopamine D_1R allowed a detailed examination of Gα-isoform specificity of these receptors (Wenzel-Seifert et al. 1999; Wenzel-Seifert and Seifert 2000; Gille and Seifert 2003). GPCR-Gα fusion proteins are also useful controls to exclude activation of Sf9 cell G proteins by a specific mammalian GPCR. Normally, the turnover number from steady-state GTPase assays or the coupling factor from [^{35}S]GTPγS binding experiments should be around unity in fusion protein systems, corresponding to linear signaling. A coupling factor >1 in a GPCR-Gα fusion protein system, however, indicates additional activation of insect cell proteins.

The fusion protein approach can also be applied to generate GPCR-RGS fusion proteins. RGS proteins (regulators of G protein signaling) activate the intrinsic GTPase activity of Gα proteins. GPCR-RGS fusion proteins bring the RGS protein in close proximity to receptor and G protein. This may enhance signal intensity in

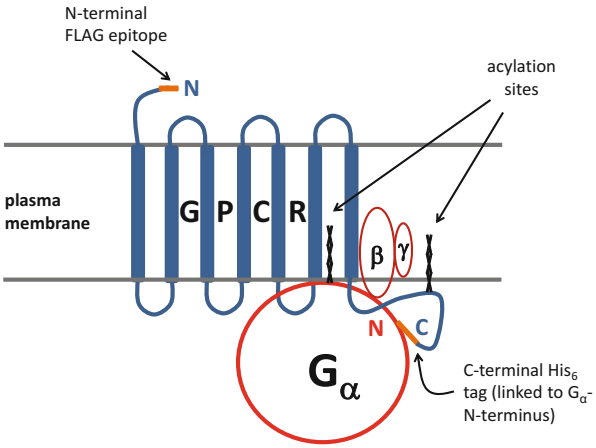

Fig. 3 Structure of a GPCR-Gα fusion protein. The GPCR is N-terminally tagged with a FLAG epitope, which allows detection by an anti-FLAG antibody, and connected to the N-terminus of a Gα-subunit via a His6 linker. Gα proteins are anchored in the plasma membrane via their acylation sites. The interaction between $G\alpha_s$ proteins and the plasma membrane is only weak in co-expression systems, but can be significantly improved in GPCR-Gαs fusion proteins

steady-state GTPase assays. The first GPCR-RGS fusion proteins were constructed in 2003 (Bahia et al. 2003). A detailed discussion of various aspects of co-expression and fusion protein systems was provided in *Methods in Enzymology* (Schneider and Seifert 2010a, b).

2 Pharmacological Characterization of Human Histamine Receptors in Sf9 Insect Cells

The biogenic amine histamine is formed by histidine decarboxylase (HDC)-mediated decarboxylation of the precursor amino acid histidine. Histamine is stored in granula of mast cells and basophils and occurs in enterochromaffin-like cells of the stomach (Panula et al. 2015). Moreover, by means of a highly sensitive HPLC-MS/MS-based detection method, histamine was identified in lymph nodes and thymus of C57Bl/6 and Balb/c mice (Zimmermann et al. 2011). In the central nervous system (CNS), histamine occurs as a neurotransmitter. It is synthesized in histaminergic neurons that emerge from the tuberomamillary nucleus (TMN) in the posterior hypothalamus and spread to numerous regions throughout the brain (Schneider et al. 2014a, b; Panula et al. 2015). The distribution of histamine in the body indicates its most important functions, namely the regulation of inflammatory/allergic reactions, stimulation of gastric acid secretion and neurotransmission. Most of the histamine effects are mediated by four G protein-coupled receptors, H_1R, H_2R, H_3R, and H_4R (Seifert et al. 2013; Panula et al. 2015). Additionally histamine acts on some non-histaminergic targets, e.g. at NMDA

receptors (Vorobjev et al. 1993; Panula et al. 2015), which, however, is not in the focus of mainstream histamine research.

This section addresses the results obtained from the pharmacological characterization of the four human histamine receptor isoforms in Sf9 cells. Other species variants will only be mentioned, when this is required by the context (e.g., comparisons of human and guinea pig H_1R or H_2R). Moreover, data from the characterization of ligands in medicinal chemistry projects will only be discussed, when they lead to new insights about structure and conformation of the corresponding receptor. Finally, publications that contain only "in silico" results without experimental verification will be omitted, since the purpose of this chapter is specifically the expression and characterization of human histamine receptors in the Sf9 cell system. For detailed information on the analysis of histamine receptor species variations in Sf9 cells or for the characterization of histamine receptor subtypes in cellular systems other than insect cells, the reader is referred to comprehensive review articles (Seifert et al. 2013; Strasser et al. 2013; Panula et al. 2015).

2.1 The Histamine H_1 Receptor

2.1.1 General Information About the Histamine H_1R

The H_1R is ubiquitously expressed, specifically in lung, CNS, and blood vessels. It preferentially couples to $G\alpha_{q/11}$ proteins, causing PLC and protein kinase C (PKC) activation as well as inositol-1,4-5-trisphosphate (IP_3) formation and intracellular Ca^{2+} release (Seifert et al. 2013; Panula et al. 2015). The typical signs of a type I allergic reaction like pruritus, increased vascular permeability, and edema are caused by H_1R activation. Therefore, administration of H_1R antagonists (so-called antihistamines) belongs to the most important anti-allergic therapeutic interventions (Simons and Simons 2011), e.g. for the treatment of allergic rhinitis. The H_1R is expressed on various types of immune cells, specifically on T cell subsets and dendritic cells and influences T cell polarization (Neumann et al. 2014). Moreover, as indicated by results from H_1R-deficient mice, the H_1R plays a role in various models of inflammatory diseases, e.g. nasal allergy, Th2-driven allergic asthma, atopic dermatitis or experimental autoimmune encephalitis (EAE) (Neumann et al. 2014). In the CNS, H_1R is involved in the regulation of locomotor activity, emotions, cognitive functions, arousal, sleep and circadian rhythm or pain perception (Schneider et al. 2014a). Moreover, the H_1R participates in the modulation of energy consumption, food intake, and respiration. H_1R blockade with antagonists increases susceptibility to seizures (Schneider et al. 2014a). Sedation, the most important side effect of brain-penetrating first-generation antihistamines, is caused by antagonism at H_1R in the CNS (Simons and Simons 2011; Neumann et al. 2014). The human H_1R (hH_1R) is endogenously expressed by various human cell lines. HeLa cervix carcinoma cells as well as U373 MG astrocytoma cells are used since more than two decades to study hH_1R pharmacology and signal transduction (Seifert et al. 2013). In the following, the

results from the characterization of the human H_1R in the Sf9 insect cell expression system will be discussed.

2.1.2 Characterization of the hH_1R in Sf9 Cell Membranes

The hH_1R was extensively characterized in Sf9 cells with regard to ligand pharmacology, and activation of G proteins. Moreover, the pharmacological differences between the hH_1R and its guinea pig orthologue (gpH_1R) were addressed by mutational and molecular modeling studies. An overview of the most important results is provided in Table 1.

Although Sf9 cells contain an endogenous PLC-stimulating $G\alpha_q$-like protein (Hepler et al. 1993), histamine does not induce a significant rise in steady-state GTPase activity in Sf9 cell membranes expressing the hH_1R alone (Houston et al. 2002). Only co-expression of the hH_1R with the regulators of G protein signaling RGS4 and GAIP (G-alpha-interacting protein, RGS19) unmasks an interaction of hH_1R with insect cell Gαq, resulting in histamine-induced stimulatory effects of 142% (RGS4) and 126% (GAIP) (Houston et al. 2002). These results indicate that the intrinsic GTPase activity of Sf9 cell $G\alpha_q$ is rate-limiting for hH_1R-mediated G protein activation in Sf9 cell membranes. This is probably due to a low number of G proteins relative to hH_1R molecules. RGS proteins commonly accelerate the intrinsic GTPase activity of Gα proteins, which results in a higher turnover and in increased availability of inactive GDP-bound Gα subunits (Fig. 2).

Due to its favorable properties, the Sf9 cell hH_1R/RGS protein co-expression system is routinely used to characterize affinity (radioligand binding), activity (steady-state GTPase assays), and binding mode of hH_1R ligands in medicinal chemistry projects. This revealed major pharmacological differences between H_1R species isoforms. Specifically, some agonistic bulky 2-phenylhistamines and histaprodifens exhibited increased efficacy and up to tenfold higher potency at gpH_1R as compared to hH_1R (Seifert et al. 2003). Such differences were also observed for antagonists. Most notably, the potency of several arpromidine-type H_1R antagonists was up to tenfold higher at gpH_1R than at hH_1R (Seifert et al. 2003). Mutagenesis experiments were performed to elucidate the molecular basis of these pharmacological species differences. Basing on the hypothesis that smaller amino acid substitutions render the gpH_1R binding pocket more flexible than the corresponding site at the hH_1R, the amino acids 153 or 433 of the hH_1R were mutated into "gpH_1R direction" (Phe-153 → Leu 153 or Ile-433 → Val 433) (Seifert et al. 2003). Although this attempt was unsuccessful in terms of generating gpH_1R-like pharmacology, the mutations dramatically decreased hH_1R receptor expression, function, electrophoretic mobility as well as [^{3}H]mepyramine (tritiated 2-((2-(Dimethylamino)ethyl)(p-methoxybenzyl)amino)-pyridine) affinity, suggesting that these amino acid positions are essential for correct folding and expression of the H_1R (Seifert et al. 2003). In addition, the hH_1R-F153L/I433V double mutant was studied. Although this protein was excellently expressed in Sf9 cell membranes, there were only partial changes in pharmacology. Thus, Phe-153 and Ile-433 cannot fully explain the species difference between hH_1R and gpH_1R (Seifert et al. 2003).

Table 1 Overview on the pharmacological characterization of the human histamine H_1R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
Only hH_1R	No histamine-induced signal in steady-state GTPase assays → no interaction with Sf9 cell G proteins	Houston et al. (2002)
hH_1R + RGS4 hH_1R + GAIP	• Interaction of hH_1R with insect cell $G\alpha_q$ unmasked • Intrinsic GTPase activity of Sf9 cell $G\alpha_q$ is rate-limiting for hH_1R-mediated G protein activation in Sf9 cell membranes. • Histamine-induced stimulation in steady-state GTPase assay: 142% with RGS4 and 126% with GAIP	
hH_1R, gpH_1R + RGS4 or GAIP	• Higher efficacy and up to tenfold higher potency of bulky 2-phenylhistamines and histaprodifens at gpH_1R than at hH_1R • Potency of several arpromidine-type H_1R antagonists up to tenfold higher at gpH_1R than at hH_1R	Seifert et al. (2003)
hH_1R-F153L[a] hH_1R-I433V[a] + RGS4 or GAIP	*Compared to wild-type hH_1R*: • Dramatic reduction of expression, function and [^{3}H]mepyramine affinity, altered electrophoretic mobility • Mutated amino acid positions required for correct folding and expression of the H_1R	
hH_1R-F153L/I433V[a] double mutant + RGS4 or GAIP	• Excellent expression, but only partial change of pharmacological properties (compared to wild-type hH_1R) • Mutated amino acid positions not solely responsible for the pharmacological difference between hH_1R and gpH_1R	
hH_1R, gpH_1R, rH_1R, bH_1R + RGS4	• Differential interaction of chiral histaprodifens with hH_1R, gpH_1R, rH_1R, and bH_1R • Two compounds showed agonism at gpH_1R, but antagonism at hH_1R, bH_1R, and rH_1R. • Potency rank order of histaprodifens: $hH_1R < bH_1R < rH_1R < gpH_1R$; structure and pharmacology of hH_1R similar to bH_1R; gpH_1R resembles rH_1R • Docking studies (active-state model of gpH_1R): multiple interaction sites between dimeric histaprodifen and gpH_1R (Asp-116, Ser-120, Lys-187, Glu-190, and Tyr-432)	Strasser et al. (2008a)
hH_1R, gpH_1R $h(gpNgpE2)H_1R$[b] $h(gpE2)H_1R$[c] + RGS4	• Higher maximum G_q-activation and lower potency of histamine at $h(gpNgpE2)H_1R$ as compared to hH_1R or $h(gpE2)H_1R$ • Differences between hH_1R and gpH_1R in N-terminus and ECL2 not responsible for pharmacological species differences • Unexpected reduction of pK_i and pEC_{50} in the series $hH_1R > h(gpE2)H_1R > h(gpNgpE2)H_1R$ for three phenoprodifens (change of ligand orientation?)	Strasser et al. (2008b)

(continued)

Table 1 (continued)

Expressed proteins	Most important new findings	Reference
hH_1R, gpH_1R $h(gpNgpE2)H_1R$ $h(gpE2)H_1R$ + RGS4	• Association rate constants for $h(gpNgpE2)H_1R$ significantly different from the constants for hH_1R and gpH_1R. • Extracellular surface of the H_1R influences ligand binding and recognition and guiding of the ligand into the binding pocket.	Wittmann et al. (2011)
hH_1R, gpH_1R, bH_1R, rH_1R + RGS4	• Identification of bulky phenylhistamines with higher potency and affinity at hH_1R than at gpH_1R • Molecular modeling: higher hH_1R potency possibly due to a more effective van der Waals interaction with $Asn^{2.61}$ of hH_1R as compared to $Ser^{2.61}$ of gpH_1R • Two distinct binding modes of phenoprodifens cause $Trp^{6.48}$ (part of the rotamer toggle switch activation mechanism) to assume either an active or an inactive conformation	Strasser et al. (2009)
hH_1R, gpH_1R + RGS4 or GAIP	N^G-acylated imidazolylpropylguanidines are partial H_1R agonists with higher efficacies at hH_1R than at gpH_1R	Xie et al. (2006a, b)

[a]Mutations were performed to make the hH_1R "more similar" to gpH_1R and to investigate the resulting alterations of receptor pharmacology
[b]Human H_1R with N-terminus and ECL2 of guinea pig H_1R
[c]Human H_1R with ECL2 of the guinea pig H_1R

A series of chiral histaprodifens was pharmacologically characterized at hH_1R and gpH_1R as well as rat (r) and bovine (b) H_1R, revealing differential interaction with H_1R species isoforms. Two of the compounds showed agonism at gpH_1R, but were antagonists at hH_1R, bH_1R, and rH_1R. The histaprodifens followed the rank order of potency $hH_1R < bH_1R < rH_1R < gpH_1R$. The hH_1R was pharmacologically and structurally similar to bH_1R, while gpH_1R resembled rH_1R (Strasser et al. 2008a). Docking studies with an active-state model of the gpH_1R and dimeric histaprodifen revealed multiple interaction sites, involving hydrogen bonds and electrostatic interactions with Asp-116, Ser-120, Lys-187, Glu-190 and Tyr-432 (Strasser et al. 2008a).

Since the amino acid sequence of the N-terminus and the second extracellular loop (ECL2) exhibit major differences between hH_1R and gpH_1R, it was hypothesized that these structures may be responsible for the preferred binding of bulky agonists to gpH_1R as compared to hH_1R. To address this hypothesis, wild-type hH_1R and gpH_1R as well as the chimeric receptors $h(gpE2)H_1R$ (hH_1R with ECL2 from gpH_1R) and $h(gpNgpE2)H_1R$ (hH_1R with N-terminus and ECL2 from gpH_1R) were co-expressed with RGS4 in Sf9 cells and compared in radioligand binding and steady-state GTPase assays (Strasser et al. 2008b). A small inverse agonistic effect of mepyramine suggests that all four receptors show only low constitutive activity. Histamine potency in steady-state GTPase assays decreased in the series $hH_1R > h(gpE2)H_1R > h(gpNgpE2)H_1R$. Maximum G_q-protein activation by histamine and the $\Delta V_{max}/B_{max}$ ratio (turnover number) was significantly enhanced at

h(gpNgpE2)H_1R as compared to hH_1R, gpH_1R, and h(gpE2)H_1R, despite a very low expression level of h(gpNgpE2)H_1R. This indicates that histamine induces a h(gpNgpE2)H_1R conformation which is specifically efficient at activating G proteins (Strasser et al. 2008b). Molecular dynamics simulations suggest that the replacement of N-terminus and ECL2 affect the network of hydrogen bonds between N-terminus, ECL1 and ECL2 and alter the conformation and flexibility of ECL2. Thus, either the replacement of the N-terminus or the combined exchange of N-terminus and ECL2 induces conformational alterations that increase the stimulatory effect of histamine and reduce its potency (Strasser et al. 2008b).

The hypothesis that major differences of N-terminus and ECL2 cause the distinct pharmacology of hH_1R and gpH_1R, however, had to be rejected, since neither binding assays nor steady-state GTPase assays revealed more pronounced "gpH_1R-like" properties of h(gpNgpE2)H_1R and h(gpE2)H_1R (Strasser et al. 2008b). Instead, three members of a new class of histaprodifens (phenoprodifens) even exhibited a *reduction* of pK_i and pEC_{50} values in the series hH_1R > h(gpE2)H_1R > h(gpNgpE2)H_1R (Strasser et al. 2008b). Previous molecular dynamics simulations with these compounds had suggested that they can adopt two distinct orientations in the gpH_2R binding pocket (Strasser et al. 2008a). Thus, the data may be explained by a change in ligand orientation in the series hH_1R – h(gpE2)H_1R – h(gpNgpE2)H_1R. Such changes, however, are probably determined early in ligand binding, which can only be addressed by kinetic binding studies (Strasser et al. 2008b).

Such experiments were performed with the antagonist [^{3}H]mepyramine and the partial agonist phenoprodifen using Sf9 cell membranes expressing RGS4 together with hH_1R, gpH_1R as well as the chimeric receptors h(gpNgpE2)H_1R and h(gpE2)H_1R (Wittmann et al. 2011). With regard to the association rate constant, h(gpNgpE2)H_1R significantly differed from both hH_1R and gpH_1R. Molecular dynamics simulations helped to explain, how the extracellular surface of the H_1R influences ligand binding kinetics, recognition of the ligand and guiding of the ligand into the binding pocket (Wittmann et al. 2011).

There are also exceptions, where bulky agonists do not interact more efficiently with gpH_1R than with hH_1R. Specifically, N^G-acylated imidazolylpropylguanidines (AIPGs) are partial H_1R agonists that exhibit higher efficacies at hH_1R as compared to gpH_1R (Xie et al. 2006a, b). Moreover, another study addressing the pharmacology of phenylhistamines and phenoprodifens at human, guinea pig, bovine, and rat H_1R identified bulky phenylhistamines with higher potency and affinity at hH_1R as compared to gpH_1R (Strasser et al. 2009). A comparison of the hypothesized binding modes of these compounds with the binding mode of the previously characterized N^G-acylated imidazolylpropylguanidine UR-AK57 (N^1-(3-Cyclohexylbutanoyl)-N^2-[3-(1*H*-imidazol-4-yl)propyl]guanidine) (Xie et al. 2006b) suggests that the higher potency at the hH_1R is caused by a more pronounced van der Waals interaction with $Asn^{2.61}$ of hH_1R as compared to $Ser^{2.61}$ of gpH_1R (Strasser et al. 2009). Moreover, phenoprodifens seem to adopt two distinctly oriented binding modes that cause the highly conserved $Trp^{6.48}$, which is part of the toggle switch mechanism of GPCR activation (Shi et al. 2002), to assume either an active or an inactive conformation (Strasser et al. 2009).

2.2 The Histamine H_2 Receptor

2.2.1 General Information About the Histamine H_2R

The H_2R is ubiquitously expressed, most importantly in stomach, heart, and CNS (Seifert et al. 2013; Schneider et al. 2014a; Panula et al. 2015). Agonist binding to this receptor results in activation of $G\alpha_s$-proteins that stimulate the adenylyl-cyclase-mediated production of the second messenger cAMP (Panula et al. 2015). The central role of the H_2R in the regulation of gastric acid production is the basis for the therapeutic use of H_2R antagonists to treat gastroesophageal reflux disease (Schubert and Peura 2008). The function of the H_2R in the brain is less well documented as for H_1R, but includes, e.g. modulation of cognitive processes and of circadian rhythm (Schneider et al. 2014a). Moreover, H_2R influences glucose metabolism and food intake (Schneider et al. 2014a).

Experiments with knockout mice have revealed that the histamine H_2R is involved in the regulation of immune responses, specifically in the modulation of Th1- or Th2-cell polarization. It should be noted, however, that the analysis of H_2R-deficient mice yields conflicting results, probably because of the variability of the disease models studied (Neumann et al. 2014). The human histamine H_2R (hH_2R) has been pharmacologically characterized in both human cells and in the Sf9 cell expression system (Seifert et al. 2013). Neutrophils are specifically well suited for the analysis of hH_2R pharmacology, because they are primary cells that can be easily isolated from human blood in large numbers. The hH_2R inhibits superoxide anion production induced by chemotactic peptides in neutrophils (Burde et al. 1989, 1990; Reher et al. 2012a) and eosinophils (Reher et al. 2012a). Moreover, H_2R activation induces functional differentiation of HL-60 promyelocytes (Klinker et al. 1996). Furthermore, it is discussed that decreased hH_2R function may contribute to inflammation in bronchial asthma (Seifert et al. 2013).

2.2.2 Characterization of the hH_2R in Sf9 Cell Membranes

The hH_2R was extensively characterized in Sf9 cells with regard to ligand pharmacology, and activation of G proteins. Moreover, the pharmacological differences between the hH_2R and its guinea pig orthologue (gpH_2R) were addressed by mutational and molecular modeling studies. An overview of the most important results is provided in Table 2.

Functional expression of the human hH_2R in Sf9 cells requires $G\alpha_s$ proteins as intracellular coupling partners. Indeed, Sf9 cells express endogenous $G\alpha_s$ proteins and activation of Sf9 cell $G\alpha_s$ has been reported for mammalian GPCRs, e.g. the bradykinin B2 receptor (Shukla et al. 2006), the LH/CG receptor (Narayan et al. 1996), or the histamine H_2R (Kühn et al. 1996). Mostly, however, the interaction of mammalian GPCRs with Sf9 cell $G\alpha_s$ shows only low productivity, which is most likely due to rapid dissociation of the activated $G\alpha_s$ subunit from the plasma membrane. Redistribution of stimulated $G\alpha_s$ proteins has been investigated in more detail in S49 lymphoma cells treated with the β-AR agonist isoproterenol (Ransnäs et al. 1989).

Table 2 Overview on the pharmacological characterization of the human histamine H_2R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
hH_2R-$Gs\alpha_S$ gpH_2R-$Gs\alpha_S$	Some H_1R agonists distinguish between H_2R species isoforms (steady-state GTPase assay)	Seifert et al. (2003)
hH_2R-$Gs\alpha_L$ hH_2R-$Gs\alpha_S$	*Comparison of hH_2R- $Gs\alpha_L$ and hH_2R- $Gs\alpha_S$*: • Similar expression level and [^{3}H]tiotidine binding • B_{max} from ligand-regulated [^{35}S]GTPγS binding >> B_{max} from [^{3}H]tiotidine binding → large part of fusion proteins not radiolabeled • GDP/GTPγS exchange velocity: hH_2R-$G_{s\alpha L}$ > hH_2R-$G_{s\alpha S}$ • Similar constitutive activity; comparable pharmacological properties of partial/inverse agonists	Wenzel-Seifert et al. (2001)
hH_2R hH_2R-$Gs\alpha_S$	• Only hH_2R: no agonist-induced signal in steady-state GTPase assays, not even with GAIP • AC activation by hH_2R (Sf9 Gαs) and by hH_2R-$Gs\alpha_S$ • No activation of insect cell or co-expressed mammalian $G\alpha_q$ by hH_2R in Sf9 cells • Mammalian $G\alpha_q$ most likely inactive in Sf9 cells	Houston et al. (2002)
hH_2R-$Gs\alpha_S$ gpH_2R-$Gs\alpha_S$	• Affinity of large guanidine-type agonists in [^{3}H]tiotidine binding: hH_2R-$Gs\alpha_S$ < gpH_2R-$Gs\alpha_S$ • Disruption of guanidine-type agonist high-affinity binding by GTPγS more effective at hH_2R-$Gs\alpha_S$ than at gpH_2R-$Gs\alpha_S$ • Potencies and efficacies of guanidines in steady-state GTPase assays: gpH_2R-$Gs\alpha_S$ > hH_2R-$Gs\alpha_S$	Kelley et al. (2001)
hH_2R-$Gs\alpha_S$ gpH_2R-$Gs\alpha_S$ hH_2R-A271D-$Gs\alpha_S$ NgpChH_2R-$Gs\alpha_S$[a] NhCgpH_2R-$Gs\alpha_S$[b]	• Higher (more "gpH_2R-like") potencies of guanidines in steady-state GTPase assays at hH_2R-A271D-$Gs\alpha_S$ and NhCgpH_2R-$Gs\alpha_S$ than at hH_2R-$Gs\alpha_S$ • Efficacies of guanidine-type agonists at hH_2R-$Gs\alpha_S$, hH_2R-A271D-$Gs\alpha_S$, NgpChH_2R-$Gs\alpha_S$ and NhCgpH_2R-$Gs\alpha_S$ are lower than at gpH_2R-$Gs\alpha_S$ • Potency and efficacy are independent H_2R properties	
hH_2R-$Gs\alpha_S$ gpH_2R-$Gs\alpha_S$ hH_2R-C17Y-$Gs\alpha_S$ hH_2R-C17Y-A271D-$Gs\alpha_S$	• Potencies and efficacies of guanidines in steady-state GTPase assays with hH_2R-C17Y-A271D-$Gs\alpha_S$: higher than at hH_2R-$Gs\alpha_S$, but lower than at gpH_2R-$Gs\alpha_S$ → Tyr-17/Asp-271 interaction not solely responsible for h/gp species differences • Possibly stabilization of ligand-specific receptor conformations • hH_2R-C17Y-$Gs\alpha_S$: basal AC activity and agonist-induced steady-state GTPase activity reduced (impaired G protein coupling or degradation of $Gs\alpha_S$?)	Preuss et al. (2007b)

(continued)

Table 2 (continued)

Expressed proteins	Most important new findings	Reference
hH_2R-gpE2-$Gs\alpha_S$[c] gpH_2R-hE2-$Gs\alpha_S$[d]	Pharmacology of guanidines in steady-state GTPase assays not significantly changed by the mutations → interaction of the mutated residues with the guanidine-binding pocket unlikely	Preuss et al. (2007c)
hH_2R-K173A-$Gs\alpha_S$ hH_2R-K175A-$Gs\alpha_S$	• Neither Lys-173- nor Lys-175 influence agonist binding in the hH_2R • Significantly lower histamine-induced steady-state GTPase signals of hH_2R-K173A-$Gs\alpha_S$ or hH_2R-K175A-$Gs\alpha_S$ → Lys173 and Lys175 important for $Gs\alpha_S$ activation?	

[a]Sequence from N-terminus to TM3 from gpH_2R and sequence from TM4 to C-terminus from hH_2R
[b]Sequence from N-terminus to TM3 from hH_2R and sequence from TM4 to C-terminus from gpH_2R
[c]Four e2 amino acids of hH_2R exchanged by the corresponding residues of gpH_2R
[d]Four e2 amino acids of gpH_2R exchanged by the corresponding residues of hH_2R

Fusion of a GPCR to $G\alpha_s$ keeps the G protein at the cell membrane and largely enhances G protein activation. This approach was used for the human histamine H_2R, which was expressed in Sf9 cells as a fusion protein with the long ($G_{s\alpha L}$) or short ($G_{s\alpha S}$) splice variant of $G\alpha_s$ (Wenzel-Seifert et al. 2001). Both fusion proteins were expressed at a similar level in Sf9 cell membranes and the affinity of the radiolabeled H_2R agonist [^{3}H]tiotidine (tritiated 1-cyano-3-[2-[[2-(diaminomethylideneamino)-1,3-thiazol-4-yl]methylsulfanyl]ethyl]-2-methyl-guanidine) was comparable (~ 32 nM) for hH_2R-$Gs\alpha_L$ and hH_2R-$Gs\alpha_S$ (Wenzel-Seifert et al. 2001). Unexpectedly, the B_{max} values of ligand-regulated [^{35}S]GTPγS binding for hH_2R-$Gs\alpha_L$ or hH_2R-$Gs\alpha_S$ exceeded the B_{max} value from [^{3}H]tiotidine binding by ~tenfold, which suggests that a large subpopulation of fusion proteins is not labeled by the radioligand (Wenzel-Seifert et al. 2001).

$G_{s\alpha L}$ exhibits lower GDP affinity than $Gs\alpha_S$, and therefore, the β_2AR-$G_{s\alpha L}$ fusion protein shows higher constitutive activity than β_2AR-$Gs\alpha_S$ (Seifert et al. 1998b). Similarly, the hH_2R-$Gs\alpha_L$ fusion protein exhibited a faster GDP/GTPγS exchange than hH_2R-$Gs\alpha_S$. Surprisingly, however, unlike the corresponding β_2AR fusion proteins, hH_2R-$Gs\alpha_L$ and hH_2R-$Gs\alpha_S$ showed similar constitutive activity and comparable pharmacological properties of partial agonists and inverse agonists in steady-state GTPase and [^{35}S]GTPγS binding assays (Wenzel-Seifert et al. 2001). This illustrates that the GDP affinity of G proteins does not influence the constitutive activity of all GPCRs to the same extent (Wenzel-Seifert et al. 2001).

It has been reported that the rH_2R couples to insect cell $G\alpha_q$ and increases intracellular Ca^{2+} in Sf9 cells (Kühn et al. 1996). However, this effect could not be confirmed and was also not observed with hH_2R or gpH_2R (Houston et al. 2002). Moreover, co-expressed GAIP did not unmask a potential interaction of hH_2R with insect cell $G\alpha_q$ (steady-state GTPase assays) although this approach was successful

with hH_1R (Houston et al. 2002). The hH_2R did not even activate mammalian $G\alpha_q$ co-expressed in Sf9 cells or fused to the hH_2R (Ca^{2+} assays, high-affinity agonist binding and [^{35}S]GTPγS binding) (Houston et al. 2002). Surprisingly, not even the hH_1R was able to activate co-expressed mammalian $G\alpha_q$ in Sf9 cells. Thus, mammalian $G\alpha_q$ was probably inactive in Sf9 cells, despite high expression levels, and therefore, Sf9 cells are not suited to investigate the interaction of GPCRs with mammalian $G\alpha_q$ (Houston et al. 2002).

When only hH_2R was expressed in Sf9 cells, no ternary complex formation with insect cell $G\alpha_s$ was observed in high-affinity agonist binding with [^{3}H]tiotidine (effect of GTPγS on histamine competition curve) and in [^{35}S]GTPγS binding (characterization of the stimulatory effect of histamine). Surprisingly, however, AC assays clearly indicated hH_2R-mediated activation of insect cell $G\alpha_s$. Thus, AC assays probably exhibit higher sensitivity than [^{3}H]tiotidine high-affinity agonist binding or [^{35}S]GTPγS binding and detect even very low insect cell $G\alpha_s$ stimulation (Houston et al. 2002). Co-expression of hH_2R with mammalian $Gs\alpha_S$ resulted in efficient G protein interaction (high-affinity agonist binding, [^{35}S]GTPγS binding, AC assays). A further increase in interaction efficiency was observed for the hH_2R-$Gs\alpha_S$ fusion protein (Houston et al. 2002).

The fusion protein approach was also used for the pharmacological comparison of hH_2R and gpH_2R (Kelley et al. 2001). In [^{3}H]tiotidine radioligand binding assays, the hH_2R-$Gs\alpha_S$ fusion protein expressed in Sf9 cells bound large guanidine-type agonists with lower affinity than gpH_2R-$Gs\alpha_S$. Moreover, GTPγS disrupted high-affinity binding of guanidine-type agonists at hH_2R-$Gs\alpha_S$ more efficiently than at gpH_2R-$Gs\alpha_S$. This indicates that the guanidine-stabilized conformation of gpH_2R interacts more tightly with the tethered G protein than the corresponding conformation of hH_2R (Kelley et al. 2001). In steady-state GTPase assays, the potencies and efficacies of guanidines were also higher with gpH_2R-$Gs\alpha_S$ than with hH_2R-$Gs\alpha_S$. However, the species isoforms did not differ in case of small agonists or antagonists (Kelley et al. 2001).

Based on molecular modeling data (bovine rhodopsin-based alignment), it was hypothesized that the high potency of guanidine-type agonists at gpH_2R is caused by the non-conserved Asp-271 in TM7 (Ala-271 in hH_2R). This hypothesis was tested by expressing the mutant hH_2R-A271D-$Gs\alpha_S$ as well as the chimeras $NgpChH_2R$-$Gs\alpha_S$ (N-terminus – TM3 from gpH_2R and TM4-C-terminus from hH_2R, containing Ala-271) and $NhCgpH_2R$-$Gs\alpha_S$ (N-terminus – TM3 from hH_2R, and TM4-C-terminus from gpH_2R, containing Asp-271) in Sf9 cell membranes (Kelley et al. 2001). In fact, steady-state GTPase assay data clearly showed increased potency of guanidines at both hH_2R-A271D-$Gs\alpha_S$ and $NhCgpH_2R$-$Gs\alpha_S$, confirming the importance of Asp-271 in the gpH_2R for guanidine binding. Unexpectedly, the efficacies of guanidine-type agonists at hH_2R-$Gs\alpha_S$ and $NgpChH_2R$-$Gs\alpha_S$ as well as the more "gpH_2R-like" constructs hH_2R-A271D-$Gs\alpha_S$ and $NhCgpH_2R$-$Gs\alpha_S$ were lower than at gpH_2R. This demonstrates that potency and efficacy are independent properties of the H_2R. The modeling and experimental data suggest that an interaction between TM1 (Tyr-17) and TM7 (Asp-271) is important for the stabilization of the guanidine-induced agonistic conformation of the gpH_2R and therefore for guanidine efficacy.

This interaction is absent in hH_2R and in the other constructs analyzed by Kelley et al. (2001).

The hypothesis that a Tyr-17/Asp-271 interaction in the gpH_2R molecule stabilizes an active receptor conformation and increases efficacy of guanidine-type agonists was tested by characterizing the mutant fusion proteins hH_2R-C17Y-$Gs\alpha_S$ and hH_2R-C17Y-A271D-$Gs\alpha_S$ (Preuss et al. 2007b). As expected, the potencies and efficacies of guanidines in the steady-state GTPase assay were higher at the hH_2R-C17Y-A271D-$Gs\alpha_S$ double mutant as compared to the wild-type hH_2R-$Gs\alpha_S$ fusion protein, but they were still below the values determined for wild-type gpH_2R-$Gs\alpha_S$. Thus, the Tyr-17/Asp-271 interaction is probably not solely responsible for the different pharmacology of hH_2R and gpH_2R (Preuss et al. 2007b). Moreover, the data suggest the stabilization of ligand-specific receptor conformations by agonists and inverse agonists in wild-type and mutant hH_2R-$Gs\alpha_S$ fusion proteins (Preuss et al. 2007b).

The results from the analysis of the hH_2R-C17Y-$Gs\alpha_S$ single mutant support the notion that an H-bond between Tyr-17 and Asp-271 stabilizes an active receptor conformation (Preuss et al. 2007b). The hH_2R-C17Y-$Gs\alpha_S$ fusion protein exhibits lower basal AC and decreased agonist-induced GTPase activities (Preuss et al. 2007b), indicating impaired G protein coupling. One possible explanation may be degradation of the hH_2R-C17Y-$Gs\alpha_S$ fusion protein in the Sf9 cells. This is suggested by the apparent molecular mass of 40 kDa instead of the expected ~80 kDa in Western blots (Preuss et al. 2007b).

In bovine rhodopsin (Palczewski et al. 2000) as well as in various aminergic GPCRs, e.g. dopamine D_2R (Shi and Javitch 2002), adenosine $A_{2a}R$ (Kim et al. 1996), or muscarinic M_3 receptor (Scarselli et al. 2007), residues in the second extracellular loop, ECL2, probably contribute to ligand binding. Thus, it was hypothesized that differences in e2 may also determine the distinct pharmacology of hH_2R-$Gs\alpha_S$ and gpH_2R-$Gs\alpha_S$ (Preuss et al. 2007c). This hypothesis was addressed by generating mutant fusion proteins with the four e2 amino acids of hH_2R exchanged by the corresponding residues of gpH_2R (hH_2R-gpE2-$Gs\alpha_S$) and vice versa (gpH_2R-hE2-$Gs\alpha_S$). Steady-state GTPase assays, however, revealed that this exchange of ECL2 did not significantly alter the pharmacology of the receptors. Thus, the mutated residues most likely do not interact with the guanidine-binding pocket (Preuss et al. 2007c).

In both hH_2R and gpH_2R, Cys-174 probably forms a disulfide bond with Cys-91 in TM3 and is framed by two lysines in position 173 and 175 (Preuss et al. 2007c). A homology model of the hH_2R predicted that these two lysines are located close to the binding site of guanidine-type agonists and are involved in agonist binding (Preuss et al. 2007c). Thus, the two mutated fusion proteins hH_2R-K173A-$Gs\alpha_S$ and hH_2R-K175A-$Gs\alpha_S$ were expressed in Sf9 cells and analyzed in steady-state GTPase activity assays. The results, however, indicate that these mutations were ineffective at altering potency or efficacy of small as well as bulky H_2R agonists (Preuss et al. 2007c). Interestingly, the effect of histamine on steady-state GTPase activity of both hH_2R-K173A-$Gs\alpha_S$ and hH_2R-K175A-$Gs\alpha_S$ was reduced, which

suggests that the lysines in positions 173 and 175 increase the efficiency of hH_2R-coupling to $G\alpha_s$ (Preuss et al. 2007c).

2.3 The Histamine H_3 Receptor

2.3.1 General Information About the hH_3R

The $G\alpha_{i/o}$-coupled histamine H_3R is mainly expressed on neurons and acts as a presynaptic auto- and heteroreceptor. It inhibits the release of histamine (Arrang et al. 1983, 1985), but also of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, or glutamate (Haas et al. 2008). Additionally, there is increasing evidence that H_3R is expressed postsynaptically (Ellenbroek and Ghiabi 2014), where it regulates, e.g. dopamine D_1R signaling (Ferrada et al. 2008; Brabant et al. 2009). Knockout mouse models demonstrate that the H_3R regulates numerous behaviors like locomotor activity, pain perception, food intake, memory, circadian rhythm, cognition, and anxiety (Schneider et al. 2014b). Moreover, H_3R-deficiency reduces addictive behavior in mouse models of ethanol consumption, which is probably due to the reward-inhibiting function of an increased histamine release (Vanhanen et al. 2013; Schneider et al. 2014b). This renders the H_3R an interesting target for the treatment of alcohol addiction (Nuutinen et al. 2012). Despite the decade-long research on H_3R pharmacology, only the inverse H_3R agonist pitolisant is currently used as an orphan drug to treat narcoleptic patients (Dauvilliers et al. 2013). Mouse models suggest that, in contrast to the other three histamine receptor subtypes, the H_3R does not seem to play a major role in immunological processes and inflammation (Neumann et al. 2014).

2.3.2 Characterization of the hH_3R in Sf9 Cell Membranes

There is no standard human cell culture model available that endogenously expresses hH_3R. Thus, expression and characterization of hH_3R and its species orthologues in the Sf9 insect cell system is of major importance (Schnell et al. 2010a, b; Schnell and Seifert 2010; Seifert et al. 2013; Strasser et al. 2013). Sf9 cells do not express endogenous Gαi-like protein that could interact with the corresponding mammalian GPCRs. It is, therefore, required to co-express the receptor of interest with mammalian $G\alpha_i$ and Gβγ subunits. This, however, provides the unique opportunity to freely combine $G\alpha_i$-coupled receptors with any $G\alpha_{i/o}$ isoform, allowing the characterization of $G\alpha_i$ isoform specificity of GPCRs. As described in the following sections, the pharmacology of the hH_3R was extensively characterized in Sf9 cells. An overview of the most important results is provided in Table 3.

Specificity of the hH_3R for $G\alpha_{i/o}$ Isoforms and Investigation of Protean Agonism

The hH_3R was co-expressed in Sf9 cells with $G\beta_1\gamma_2$ and $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$. All hH_3R/G protein combinations could be readily expressed in Sf9 cells, and a semiquantitative analysis of expression levels by Western blot (purified $G\alpha_{i2}$ and

Table 3 Overview on the pharmacological characterization of the human histamine H_3R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
hH_3R	No relevant stimulation of insect cell G proteins	Schnell et al. (2010a)
hH_3R + $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ + $G\beta_1\gamma_2$	• hH_3R/Gα coupling ratio between 1:2 and 1:11 • Steady-state GTPase assay: high constitutive activity of hH_3R (comparable to hH_4R) • [^{3}H]NAMH binding and steady-state GTPase assays: pharmacological properties independent of the type of co-expressed $G\alpha_{i/o}$ protein • No protean agonism of proxyfan	
hH_3R-$G\alpha_{i2}$ or hH_3R-$G\alpha_o$ + $G\beta_1\gamma_2$	• Similar pharmacological properties of hH_3R-$G\alpha_{i2}$ and hH_3R-$G\alpha_o$ (steady-state GTPase assays) → hH_3R pharmacology independent of $G\alpha_{i/o}$ isoform • No protean agonism of proxyfan	
rH_3R + $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ + $G\beta_1\gamma_2$	• rH_3R/$G\alpha_{i/o}$ coupling stoichiometry similar to hH_3R • High constitutive activity with all $G\alpha_{i/o}$ subunits • Pharmacological properties independent of co-expressed $G\alpha_{i/o}$ isoform (similar to hH_3R) • No protean agonism of proxyfan	Schnell et al. (2010b)
rH_3R or hH_3R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	*[^{3}H]NAMH binding and/or steady-state GTPase assays:* • No species selectivity of histamine, Nα-methylhistamine, (*R*)-α-methylhistamine, imetit, and clobenpropit • Striking species selectivity of imoproxifan: nearly full agonist at hH_3R, but inverse agonist at rH_3R • Imoproxifan: $pEC_{50} > pK_i$ (hH_3R and rH_3R) → conformations with low partial/inverse agonist affinity, but efficient Gα interaction?	
hH_3R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ Influence of ions on hH_3R properties	*[^{3}H]NAMH radioligand binding:* Increase in radioligand B_{max} and no significant reduction of binding affinity by 100 mM of NaCl *Effect of NaCl (100 mM) in steady-state GTPase assays:* • Increase in efficacy and reduction of potency of histamine • Reduction of efficacy and increase in potency of thioperamide → stabilization of hH_3R inactive state by NaCl *Comparison of various cations and anions:* Rank order of efficacy at inhibiting hH_3R constitutive activity: $Li^+ \sim Na^+ \sim K^+ < Cl^- < Br^- < I^-$	Schnell and Seifert (2010)
hH_3R + $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ + $G\beta_1\gamma_2$	*NaCl effect on hH_3R basal activity:* strongest NaCl-mediated reduction of constitutive activity in the presence of $G\alpha_{i3}$	

(continued)

Table 3 (continued)

Expressed proteins	Most important new findings	Reference
hH_3R-D2.50N + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	• Reduction of binding sites and lower [^{3}H]NAMH affinity (absence of NaCl) • Constitutive activity (steady-state GTPase assays) completely eliminated • Stimulatory effect of histamine still NaCl-sensitive	Schnell and Seifert (2010)
hH_3R-D2.50N + $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ + $G\beta_1\gamma_2$	Surprising G protein selectivity of hH_3R-D2.50N mutation: no interaction with $G\alpha_{i3}$, but activation of $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{o1}$	

$G\alpha_o$ as reference) yielded receptor-to-G protein ratios between 1:50 and 1:100 (Schnell et al. 2010a). The receptor expression levels determined by Western blot were confirmed by radioligand saturation binding assays with the antagonist [^{3}H]JNJ-7753707 ((4-Fluorophenyl)(1-methyl-2-{[1-(1-methylethyl)piperidin-4-yl]methoxy}-1*H*-imidazol-5-yl)methanone). By contrast, quantitation of the total number of activated $G\alpha_{i/o}$ proteins in [^{35}S]GTPγS binding assays revealed a much lower amount of [^{35}S]GTPγS binding sites as compared to the Western blot results, yielding $hH_3R/G\alpha_i$ isoform coupling ratios between 1:2 ($hH_3R/G\alpha_{i1}$) and 1:11 ($hH_3R/G\alpha_o$) (Schnell et al. 2010a).

Potencies and efficacies of the physiological agonist histamine and the inverse agonist thioperamide (*N*-Cyclohexyl-4-(imidazol-4-yl)-1-piperidinecarbothioamide) were determined in steady-state GTPase assays for all $hH_3R/G\alpha_{i/o}$ combinations (Schnell et al. 2010a). When hH_3R was expressed in Sf9 cell membranes without any mammalian G protein, the signals induced by histamine and thioperamide were only small, indicating that hH_3R-mediated stimulation of insect cell G proteins was virtually absent (Schnell et al. 2010a). A comparison of all five expression systems (hH_3R alone and combined with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$) revealed that the relative stimulatory signal of histamine and the relative inhibitory signal of thioperamide were comparable, indicating that the constitutive activity of hH_3R does not depend on the type of co-expressed $G\alpha_{i/o}$ protein (Schnell et al. 2010a). Overall, the constitutive activity of the hH_3R was similar to the basal activity of the hH_4R (Schneider et al. 2009) (see following section).

Steady-state GTPase experiments were also performed with various hH_3R standard ligands in all $hH_3R/G\alpha_{i/o}$ co-expression systems. N^{α}-methylhistamine (NAMH) and (R)-α-methylhistamine (RAMH) turned out to be full agonists under all conditions and imetit almost reached full efficacy. Proxyfan (4-[3-(Phenylmethoxy)propyl]-1*H*-imidazole) and impentamine (4-(5-Aminopentyl)imidazole) were partial agonists with comparable efficacy under all conditions. Ciproxifan (cyclopropyl-(4-(3-(1*H*-imidazol-4-yl)propyloxy)phenyl) ketone), clobenpropit (*N*-(4-Chlorobenzyl)-*S*-[3-(4(5)-imidazolyl)propyl]isothiourea), and thioperamide exhibited inverse agonism in all systems, but efficacies were significantly different between the various $G\alpha_{i/o}$ proteins. Nevertheless, the rank orders of potency and efficacy of the ligands remained unaltered. Taken together, these experiments again confirm the notion that the hH_3R

exhibits similar pharmacological properties independently of the co-expressed $G\alpha_{i/o}$ isoforms (Schnell et al. 2010a).

As mentioned above, the hH_3R/G protein ratios ranged between 1:2 and 1:11, indicating that it is difficult to exactly control the expression levels of receptor and G proteins. Thus, the fusion protein approach was used to ensure a 1:1 coupling ratio of hH_3R and Gα subunit. The hH_3R was fused to $G\alpha_{i2}$ and $G\alpha_o$, because these two $G\alpha_{i/o}$ isoforms exhibit the lowest structural similarity. The pharmacological properties of the standard ligands histamine, imetit, proxyfan, clobenpropit, and thioperamide were similar in steady-state GTPase assays with hH_3R-$G\alpha_{i2}$ and hH_3R-$G\alpha_o$. This indicates again that the hH_3R pharmacology is largely independent of the type of co-expressed or fused Gα subunit (Schnell et al. 2010a).

Previously published studies about hH_3R pharmacology had reported that, depending on the expression system and the functional readout, proxyfan can be a full, a partial, or even an inverse agonist (Gbahou et al. 2003; Krueger et al. 2005). This was explained by the phenomenon of "protean agonism," which is the ability of a ligand to induce GPCR conformations with lower G protein-coupling efficiency than the agonist-stimulated or constitutively active receptor (Gbahou et al. 2003). It has been hypothesized that protean agonism of proxyfan is due to functional selectivity, i.e. G protein coupling of the proxyfan-bound hH_3R differentiates between various $G\alpha_{i/o}$ isoforms. The data reported by Schnell et al. (2010a), however, strongly suggest that neither proxyfan nor any other of the tested hH_3R ligands exhibits this kind of functional selectivity, at least when the hH_3R is co-expressed with or fused to various $G\alpha_{i/o}$ isoforms in Sf9 cell membranes. One reason for this discrepancy could be the influence of different types of Gβγ subunits, which was not systematically investigated in Sf9 cells, because in the experiments performed by Schnell et al. (2010a) all hH_3R/$G\alpha_{i/o}$ combinations were uniformly co-expressed with $G\beta_1\gamma_2$. Moreover, specific combinations of various $G\alpha_{i/o}$ isoforms or cross-talk between signaling pathways could have influenced the results reported by Gbahou et al. (2003) and Krueger et al. (2005).

Species Differences Between Human and Rat Histamine H_3R

As discussed in the preceding section, the study of Gbahou et al. (2003) suggested that proxyfan shows protean agonism, which, however, was not confirmed in the Sf9 cell system (Schnell et al. 2010a). One of the reasons for this discrepancy could be a pharmacological difference in H_3R isoforms. Gbahou et al. (2003) used rat H_3R (rH_3R), while the experiments of Schnell et al. (2010a) were performed with hH_3R. To test this hypothesis, both species isoforms were directly compared in the Sf9 cell expression system (Schnell et al. 2010b).

Similar to the human isoform (Schnell et al. 2010a), the rH_3R was also co-expressed with $G\beta_1\gamma_2$ and the $G\alpha_{i/o}$ isoforms $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$. A quantitation of rH_3R binding sites by radioligand binding with [^{3}H]JNJ-7753707 and of receptor-coupled Gα subunits by [^{35}S]GTPγS binding revealed a rH_3R/G protein stoichiometry between 1:2 and 1:7 (Schnell et al. 2010b), which is comparable to the properties of the corresponding hH_3R membranes (Schnell et al. 2010a). Moreover, similar to the hH_3R, the rH_3R showed similar high constitutive activity with each of the four $G\alpha_{i/o}$

subunits as indicated by comparable relative effects of the agonist histamine and the inverse agonist thioperamide (Schnell et al. 2010b). The independence of rH_3R pharmacology of the co-expressed $G\alpha_{i/o}$ type was confirmed by steady-state GTPase experiments.

Several H_3R standard ligands were characterized at rH_3R (+ $G\alpha_{i2}G\beta_1\gamma_2$) and hH_3R (+ $G\alpha_{i2}G\beta_1\gamma_2$) in [^{3}H]NAMH radioligand binding assays. The affinities of histamine, N^{α}-methylhistamine, (R)-α-methylhistamine, imetit, proxifan, and clobenpropit did not differ between species isoforms, while the affinities of impentamine, imoproxifan, ciproxifan, and thioperamide were increased at the rH_3R (Schnell et al. 2010b). The radioligand binding results were largely confirmed on the functional level by steady-state GTPase experiments. Histamine, N^{α}-methylhistamine, RAMH, imetit, and clobenpropit did not show species selectivity. Impentamine, however, was more potent at rH_3R than at hH_3R. Additionally, ciproxifan and thioperamide exhibited higher potency but less efficacy at rH_3R as compared to hH_3R (Schnell et al. 2010b). The hypothesis that the protean agonism of proxyfan reported by Gbahou et al. (2003) was characteristic for the rat H_3R orthologue had to be rejected, because proxyfan acted as a strong partial agonist at rH_3R expressed in Sf9 cells, independently of the co-expressed G protein (Schnell et al. 2010b).

A striking difference between hH_3R and rH_3R was observed for the H_3R ligand imoproxifan, which acted as a nearly full agonist at the hH_3R, but exhibited inverse agonism at the rat orthologue (Schnell et al. 2010b). To explain this switch in quality of action, molecular modelling studies were performed by docking imoproxifan into the binding site of the active hH_3R and the inactive rH_3R. The simulations revealed different electrostatic surfaces between TM V and TM III. While the hH_3R shows a positive surface potential in this region (NH moiety of $Trp^{6.48}$), the corresponding part of the rH_3R is slightly negatively charged (OH moiety of $Thr^{6.52}$), which results in different orientations of the ligand at both receptors. Moreover, hH_3R differs from rH_3R in amino acid position 3.37. $Thr^{3.37}$ of the hH_3R interacts with $Glu^{5.46}$, making $Glu^{5.46}$ pointing away from the binding pocket, which creates a binding site for the imoproxifan methyl moiety (Schnell et al. 2010b). By contrast, an alanine in position 3.37 of the rH_3R precludes any electrostatic interaction between $Glu^{5.46}$ and position 3.37.

$Ala^{3.40}$ of hH_3R is replaced by the bulkier $Val^{3.40}$ in rH_3R. Thus, the imoproxifan oxime moiety points downward towards $Ala^{3.40}$ in hH_3R and stabilizes $Trp^{6.48}$ in its horizontal conformation via a hydrogen bond. By contrast, the oxime moiety is directed upwards in rH_3R and interacts with $Thr^{6.52}$, while the methyl group of imoproxifan fits into a pocket between $Val^{3.40}$ and $Trp^{6.48}$. This stabilizes $Trp^{6.48}$ of rH_3R in its vertical conformation. According to the rotamer toggle switch mechanism of GPCR activation (Shi et al. 2002), the horizontal conformation of $Trp^{6.48}$ corresponds to the active state, while the vertical conformation stabilizes the inactive receptor state. Thus, this model explains the different quality of action of imoproxifan at hH_3R and rH_3R (Schnell et al. 2010b).

Interestingly, in case of imoproxifan, a comparison of steady-state GTPase assay and [^{3}H]NAMH radioligand binding data revealed that the pEC_{50} values at hH_3R

and rH_3R were significantly higher than the corresponding pK_i values. This suggests that both hH_3R and rH_3R can adopt conformations with low affinity to partial/inverse agonists that nevertheless exhibit efficient G protein interaction (Schnell et al. 2010b).

Influence of Monovalent Ions on hH_3R Function

According to the (simplifying) two-state model of receptor activation (Fig. 4), GPCRs can adopt an active or an inactive conformation (Leff 1995). The equilibrium between both receptor states is shifted to the active side by (partial) agonists and/or interaction with G proteins. The inactive state, however, is stabilized by (partial) inverse agonists (Schneider et al. 2010b; Sato et al. 2016). The degree of constitutive activity depends on the intrinsic tendency of the receptor protein to occur in the active state. It is well established that ions are able to modulate GPCR function (Strasser et al. 2015). Specifically, sodium represents an allosteric stabilizer of the inactive receptor conformation and inhibits constitutive activity, which was, e.g., demonstrated for chemoattractant receptors (Seifert and Wenzel-Seifert 2001, 2003).

As discussed above, the hH_3R exhibits high constitutive activity. Thus, hH_3R represents an interesting model for the detailed investigation of the activity-modulating effects of ions. The hH_3R was co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cells and the influence of 100 mM of NaCl on [^{3}H]NAMH high-affinity agonist binding and on GTP hydrolysis in the steady-state GTPase assay was investigated. Unexpectedly, in contrast to the data reported for other $G\alpha_{i/o}$-coupled receptors like FPR1 (Seifert and Wenzel-Seifert 2003), the affinity of the hH_3R to the radioligand

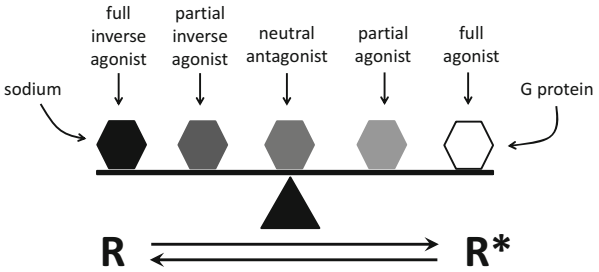

Fig. 4 Two-state model of receptor activation and factors stabilizing the active (R*) and inactive (R) receptor conformation. Every GPCR population exists in an equilibrium of active and inactive receptor conformations. Full agonists produce a maximum shift towards the active side, while inverse agonists cause a maximum stabilization of the inactive GPCR conformation. Partial agonists and partial inverse agonists induce only an incomplete shift towards either side. Neutral antagonists bind to all receptor states with the same affinity and therefore do not change the equilibrium. G proteins stabilize the active conformation, while sodium ions usually uncouple GPCRs from their G proteins by shifting the equilibrium towards the inactive side. It should be noted that, despite its usefulness, the two-state model is very simplistic and does not account for the numerous distinct ligand- and G protein-specific receptor conformations occurring in reality. Adapted from Schneider and Seifert (2010a)

was not significantly reduced by NaCl. Moreover, most surprisingly, the B_{max} value was even increased by NaCl. The NaCl resistance of the hH_3R in the [^{3}H]NAMH radioligand binding assays is not fully explained yet, but may be caused by the extremely high constitutive activity of the hH_3R (Schnell and Seifert 2010).

The resistance of the hH_3R to the effect of NaCl in radioligand binding was not reflected by the data from steady-state GTPase experiments. In the presence of 100 mM of NaCl, the efficacy of histamine (full agonist) was increased and the pEC_{50} value of histamine was reduced from 8.01 to 7.53. By contrast, the pIC_{50} value of thioperamide (inverse agonist) was increased from 7.15 to 7.43 by NaCl, while the efficacy of thioperamide was reduced. This clearly indicates that NaCl stabilizes the inactive state of the hH_3R and reduces the constitutive activity of the system, which agrees with the predictions of the two-state model system of receptor activation (Schnell and Seifert 2010).

Since NaCl does not only contain sodium cations but also chloride anions, it is not clear if the effect of NaCl on hH_3R constitutive activity is mediated by Na^+, by Cl^- or by both ions. To address this question, a profile of the effects of various monovalent cations (Li^+, Na^+, and K^+) as well as of different anions (Cl^-, Br^-, and I^-) was determined in steady-state GTPase assays with membranes expressing hH_3R plus $G\alpha_{i2}$ and $G\beta_1\gamma_2$. The rank order of efficacy was $Li^+ \sim Na^+ \sim K^+ < Cl^- < Br^- < I^-$. This indicates a direct proportionality between anion radii and reduction of basal hH_3R activity and shows that anions contribute more to the salt-induced reduction of constitutive activity than cations. Moreover, the different efficacies of the anions exclude the possibility that an increased osmolality may be responsible for the effect on constitutive activity (Schnell and Seifert 2010). Similar results had been previously obtained with the $h\beta_2AR$-$Gs\alpha_L$ fusion protein, and it had been hypothesized that anions may enhance GDP affinity to the G protein, reducing the ability of the receptor to promote GDP dissociation (Seifert 2001). Interestingly, a comparison of the NaCl effect on hH_3R basal activity in membranes co-expressing $G\beta_1\gamma_2$ and various $G\alpha_{i/o}$ subunits ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_{i/o}$) revealed the strongest NaCl-mediated reduction of constitutive activity in the presence of $G\alpha_{i3}$ (Schnell and Seifert 2010).

It is generally assumed that the highly conserved $Asp^{2.50}$ acts as a Na^+ binding site in GPCRs (Horstman et al. 1990; Wittmann et al. 2014). Thus, the functional consequences of a charge-neutralizing mutation from $Asp^{2.50}$ to $Asn^{2.50}$ in the hH_3R protein were investigated. In the absence of sodium, the D2.50N mutant (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) exhibited a reduced number of [^{3}H]NAMH binding sites and an affinity reduction of [^{3}H]NAMH by about 90% as compared to the wild-type hH_3R (Schnell and Seifert 2010). Constitutive activity in steady-state GTPase assays was completely eliminated by the D2.50N mutation (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) and consequently, neither thioperamide nor NaCl further inhibited basal activity. Interestingly, however, the stimulatory effect of histamine at the D2.50N mutant was highly sensitive to NaCl and was completely eliminated at NaCl concentrations > 90 mM. Most surprisingly, the D2.50N mutation introduced G protein selectivity, as the mutant did not productively interact any more with $G\alpha_{i3}$, but still activated $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{o1}$. Thus, $Asp^{2.50}$ seems to play a decisive role in the $hH_3R/G\alpha_{i3}$-interaction (Schnell and Seifert 2010). In

summary, the characterization of the hH_3R in the Sf9 cell expression system by Schnell and Seifert (2010) revealed that $G\alpha_{i3}$ interacts with hH_3R in a very distinct manner as compared to the other tested $G\alpha_{i/o}$ isoforms (stronger NaCl effect on activity of wild-type hH_3R and complete inactivity of the hH_3R-D2.50N mutant). Interestingly, the D2.50N mutant was not completely NaCl-insensitive, which indicates that the interaction between ions and hH_3R is more complex and cannot be explained by a single interaction site (Schnell and Seifert 2010).

In contrast to the hH_3R, the structurally similar hH_4R (see Sect. 2.4) exhibits completely NaCl-resistant constitutive activity (Schneider et al. 2009). A potential explanation for this discrepancy was recently offered by Wittmann et al. (2014). A comparison of various human aminergic GPCRs revealed that in the majority of receptors, glycine is the most abundant (80%) amino acid in the sodium binding channel between the ligand binding site and the sodium binding region (Wittmann et al. 2014). This is, however, not the case for hH_3R and hH_4R. Moreover, in hH_4R the glutamine in position 7.42 disrupts a water chain, which is extending from $Asp^{3.32}$ (orthosteric binding site) to $Asp^{2.50}$ (allosteric binding site). This might kinetically prevent sodium from binding to the allosteric binding site (Wittmann et al. 2014).

2.4 The Histamine H_4 Receptor

The fourth histamine receptor couples to PTX-sensitive $G\alpha_i$ proteins, specifically to $G\alpha_{i2}$ and shows high constitutive activity (Schneider et al. 2009). The H_4R is a chemotactic receptor mainly expressed on hematopoietic cells, specifically on eosinophils (O'Reilly et al. 2002; Buckland et al. 2003; Reher et al. 2012b). Human eosinophils belong to the best characterized primary cells endogenously expressing hH_4R, but it is difficult to isolate this rare cell type in sufficiently high purity and numbers from healthy volunteers (Seifert et al. 2013). Moreover, H_4R is expressed on mast cells (Hofstra et al. 2003; Jemima et al. 2014) as well as dendritic cells (Gutzmer et al. 2005; Damaj et al. 2007; Bäumer et al. 2008; Gschwandtner et al. 2011) and expression on natural killer cells has been reported, too (Damaj et al. 2007). The presence of the H_4R on monocytes is discussed controversially (Damaj et al. 2007; Gschwandtner et al. 2013; Werner et al. 2014). Data from a comprehensive analysis of hH_4R expression on various myeloid cell types have been published very recently (Capelo et al. 2016). H_4R knockout mouse models suggest that this receptor plays a role in the pathophysiology of itch, experimental asthma and EAE (Neumann et al. 2014).

The H_4R represents an interesting target for anti-inflammatory drugs. For example, the H_4R regulates eosinophilic inflammation in a mouse model of ovalbumin-induced allergic asthma (Hartwig et al. 2015). Moreover, the hH_4R seems to be a key player in pruritus during inflammatory reactions (Bell et al. 2004; Dunford et al. 2007; Rossbach et al. 2011). However, studies with mouse models should be interpreted with caution, because H_4R pharmacology strongly differs between various species (Strasser et al. 2013). For example, the "prototypical" hH_4R antagonist JNJ7777120 (1-[(5-Chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine) is an inverse

agonist at the hH_4R, but a partial agonist at the rat, mouse, and canine orthologues (Schnell et al. 2011; Strasser et al. 2013). Another caveat is H_4R-induced G protein-independent β-arrestin signaling. Although JNJ-7777120 is an inverse H_4R agonist with regard to G protein activation, it exhibits agonistic effects on H_4R-dependent β-arrestin signaling (Rosethorne and Charlton 2011; Seifert et al. 2011; Nijmeijer et al. 2013). Recently, the H_4R antagonist JNJ 39758979 ((R)-4-(3-amino-pyrrolidin-1-yl)-6-isopropyl-pyrimidin-2-ylamine) was shown to be safe and efficacious at reducing histamine-induced pruritus in a phase 1 clinical study (Kollmeier et al. 2014).

2.4.1 Successful Reconstitution of Functional Human Histamine H_4R (hH_4R) in Sf9 Cells

The N-terminally FLAG-tagged and C-terminally His-tagged wild-type hH_4R was co-expressed with $G\alpha i_2$ and $G\beta_1\gamma_2$ in Sf9 cells. Binding studies with [^{3}H]histamine revealed a K_D value of ~10 nM (Schneider et al. 2009), which fits well to the literature range (5–20 nM). Steady-state GTPase and [^{35}S]GTPγS binding experiments confirmed the high constitutive activity of the hH_4R, which was effectively inhibited by the inverse agonist thioperamide (Schneider et al. 2009). Surprisingly, thioperamide was not able to suppress [^{35}S]GTPγS binding in the co-expression system (hH_4R + $G\alpha i_2$ + $G\beta_1\gamma_2$) to the level of control membranes expressing only $G\alpha i_2$ and $G\beta_1\gamma_2$ (Schneider et al. 2009). This strongly indicates that thioperamide is only a partial H_4R inverse agonist and not, as originally suggested in the literature (Lim et al. 2005), a full inverse agonist. The Sf9 cell system provides a "clean" background devoid of mammalian $G\alpha_i$ proteins and their cognate GPCRs. Thus, expression of mammalian G proteins without GPCRs in Sf9 cells provides a valid control for baseline Gα activity and for the maximum possible effect of a full inverse agonist. In the following, the most important results from the pharmacological characterization of the hH_4R in Sf9 cell membranes are discussed. An overview of the most important results is provided in Table 4.

2.4.2 G Protein-Independent High-Affinity-State of the hH_4R

According to the ternary complex model (De Lean et al. 1980), a GPCR shows its highest agonist affinity, when it is part of the ternary complex (Sect. 1.2.1, Fig. 2). Ternary complex formation, however, is prevented in the presence of GTPγS which binds to the Gα subunit like GTP (Gilman 1987), but cannot be hydrolyzed. Thus, GTPγS disrupts the G protein cycle, resulting in the accumulation of uncoupled inactive GPCRs with reduced agonist affinity. Surprisingly the hH_4R shows an active state which is completely independent of G proteins (Schneider et al. 2009). This is supported by the following four observations: First, high-affinity [^{3}H]histamine binding (K_D and B_{max}) to membranes expressing hH_4R, $G\alpha_{i2}$, and $G\beta_1\gamma_2$ was retained in the presence of GTPγS. Second, [^{3}H]histamine binding affinity was almost identical in the $hH_4R/G\alpha_{i2}/G\beta_1\gamma_2$ co-expression system and in Sf9 cell membranes expressing hH_4R in the absence of mammalian G proteins. Third, the K_i values of the inverse hH_4R agonists thioperamide and JNJ-7777120 were unaltered in membranes expressing only hH_4R, although the two-state model of receptor activation (Fig. 4) suggests that inverse agonist affinity increases, when

Table 4 Overview on the pharmacological characterization of the human histamine H_4R in the Sf9 cell expression system

Expressed proteins	Most important new findings	Reference
hH_4R	• GTPγS-insensitive high-affinity agonist binding • No activation of insect cell G proteins	Schneider et al. (2009)
$hH_4R + G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o + G\beta_1\gamma_2$	▪ hH_4R most efficiently activates $G\alpha_{i2}$ ▪ Least efficient interaction with $G\alpha_o$	
$hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$	• GTPγS-insensitive high-affinity agonist binding • Very high sodium-insensitive constitutive activity • Structural instability • Catalytic signaling	
hH_4R-$G\alpha_{i2} + G\beta_1\gamma_2$	• Compared to co-expression system ($hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$): • Increased expression level • Unaltered histamine affinity • More efficient $hH_4R/G\alpha_{i2}$ interaction • Increased constitutive activity • Linear signaling	
hH_4R-A6.30E + $G\alpha_{i2} + G\beta_1\gamma_2$	*Compared to wild-type* *($hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$)*: • Slight (non-significant) reduction of constitutive activity and G protein coupling efficiency • Unaltered K_D of histamine • G protein-independent high-affinity binding retained	Schneider et al. (2010a)
hH_4R-R3.50A + $G\alpha_{i2} + G\beta_1\gamma_2$	*Compared to wild-type* *($hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$)*: • G protein coupling eliminated • Affinity of thioperamide increased • Affinity of histamine reduced	
hH_4R + RGS4 + $G\alpha_{i2} + G\beta_1\gamma_2$	*Compared to wild-type co-expression system* *(+ $G\alpha_{i2}G\beta_1\gamma_2$)*: • No significant change of histamine effect and baseline steady-state GTPase activity • Significant increase of thioperamide inverse agonistic effect	Schneider and Seifert (2009)
hH_4R-RGS4 + $G\alpha_{i2} + G\beta_1\gamma_2$	*Compared to wild-type co-expression system* *(+ $G\alpha_{i2}G\beta_1\gamma_2$)*: • Significant increase of baseline steady-state GTPase activity and of thioperamide inverse agonistic effect • Significantly increased EC_{50}-values of histamine and JNJ-7777120 (~twofold) • Significantly increased apparent K_M value of $G\alpha_{i2}$ intrinsic GTPase activity in the presence of histamine	

(continued)

Table 4 (continued)

Expressed proteins	Most important new findings	Reference
hH_4R + GAIP + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	*Compared to wild-type co-expression system (+ $G\alpha_{i2}G\beta_1\gamma_2$)*: • No significant change of signal range and baseline activity in steady-state GTPase assays • Significantly increased apparent K_M value of $G\alpha_{i2}$ intrinsic GTPase activity in the presence of histamine	
hH_4R-GAIP + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	*Compared to wild-type co-expression system (+ $G\alpha_{i2}G\beta_1\gamma_2$)*: • Basically identical pharmacological properties • Significantly increased relative histamine- and thioperamide-induced signals in steady-state GTPase assays	
hH_4R-GAIP + $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ + $G\beta_1\gamma_2$	Identical G protein selectivity of hH_4R-GAIP and hH_4R → G protein coupling is mainly determined by the GPCR, but not by the RGS protein	
hH_4R-F169V+S179A or hH_4R-F169V+S179M hH_4R-F169V hH_4R-S179A hH_4R-S179M + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	*Compared to wild-type hH_4R:* constitutive activity not affected by the S179A or S179M single mutations. Constitutive activity slightly reduced in F169V single mutant; stronger reduction in double mutants. S179A single mutant: increased potency and affinity of JNJ-7777120	Wifling et al. (2015b)
mH_4R-V171F mH_4R-V171F+M181S + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	No constitutive activity of wild-type mH_4R and mH_4R-V171F mutant, but weak constitutive activity of mH_4R-V171F +M181S double mutant	
hH_4R-F168A + $G\alpha_{i2}$ + $G\beta_1\gamma_2$	Total loss of hH_4R constitutive activity.	Wifling et al. (2015a)

the receptor is not coupling to G proteins and assumes an inactive state. Finally, steady-state GTPase assays with membranes co-expressing hH_4R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ revealed that the constitutive activity of the hH_4R is insensitive to sodium ions. According to the standard two-state model of receptor activation depicted in Fig. 4, however, it is expected that Na^+ stabilizes the inactive state of a GPCR. This has been shown previously, e.g. for FPR-26 (Wenzel-Seifert et al. 1998; Seifert and Wenzel-Seifert 2001) or the α_2-adrenoceptor (Tian and Deth 2000).

2.4.3 Analysis of hH_4R-G Protein Coupling

Analysis of hH_4R activation in the steady-state GTPase assay in membranes co-expressing hH_4R with $G\beta_1\gamma_2$ and a specific $G\alpha$ subunit ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or

$G\alpha_o$) revealed that $G\alpha_{i2}$ was most effectively stimulated by the hH_4R. By contrast, the hH_4R hardly activated $G\alpha_o$ proteins (Schneider et al. 2009). Since $G\alpha_o$ is the main G protein subtype in the brain, this result suggests that the hH_4R is not of major importance in the CNS. We have seriously questioned the widespread but largely unfounded notion of functional hH_4R expression on neurons (Schneider and Seifert 2016).

The stoichiometry of the receptor-G protein interaction can be calculated by dividing the total number of receptor-regulated G proteins (from GTPγS binding assays) by the number of receptors per cell (B_{max} from radioligand binding or from Western blot). When co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cell membranes, the hH_4R catalytically activates up to five $G\alpha_{i2}$ subunits simultaneously (Schneider et al. 2009). The affinity of [^{35}S]GTPγS to the Gα subunit (K_D value) reflects efficiency of G protein activation. The inverse agonistic character of thioperamide was confirmed in [^{35}S]GTPγS assays with membranes co-expressing hH_4R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$. While the [^{35}S]GTPγS K_D value was 3.4 nM in the presence of histamine, it was about threefold increased by thioperamide (Schneider et al. 2009), indicating reduced [^{35}S]GTPγS affinity of the Gα subunit due to uncoupling from the hH_4R.

2.4.4 Conformational Instability of hH_4R

As demonstrated for the constitutively active mutant of the β_2-adrenoreceptor (β_2AR_{CAM}) (Gether et al. 1997), constitutive activity of a GPCR increases conformational flexibility and favors denaturation. By contrast, ligand binding reduces conformational flexibility and stabilizes the receptor. Thus, addition of ligands to a cell culture expressing β_2AR_{CAM} increased the B_{max} value of this receptor (Gether et al. 1997). This effect was caused by both agonists and inverse agonists, suggesting that it is the switch between different activation states rather than the nature of the activation state, which destabilizes the receptor.

The high constitutive activity of the hH_4R prompted us to investigate its conformational stability and the stabilizing effect of ligands. In fact, addition of histamine (10 μM) or thioperamide (1 μM) to Sf9 cells co-expressing hH_4R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ significantly increased the B_{max} value in histamine high-affinity agonist binding assays (Schneider et al. 2009). Interestingly, this effect was not visible in immunoblots, indicating that histamine and thioperamide mainly support the correct folding of hH_4R in the cell membrane, but not during intracellular protein synthesis. This was confirmed in experiments, where denaturation of hH_4R (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) was induced by incubation of the membranes at 37°C. After 120 min, almost 70% of the histamine binding sites in the ligand-free control were lost, but only 35% in the presence of histamine. Most surprisingly, however, thioperamide *increased* the B_{max} by 30–40%, suggesting that it did not only prevent hH_4R denaturation, but even re-folded a priori misfolded receptors. This intriguing "refolding" effect of the inverse agonist thioperamide was confirmed in a two-step assay, during which the receptor was first denatured and then incubated with thioperamide.

2.4.5 Characterization of the hH_4R-$G\alpha_{i2}$ Fusion Protein

To analyze the interaction of the hH_4R with $G\alpha_{i2}$, the C-terminus of the receptor was fused to the N-terminus of the G protein by using a His_6 linker (Fig. 3). The hH_4R-$G\alpha_{i2}$ protein co-expressed with $G\beta_1\gamma_2$ in Sf9 cell membranes exhibited linear signaling with a coupling factor of ~1 in [^{35}S]GTPγS binding assays and a turnover number of ~1 in steady-state GTPase assays. Thus, hH_4R exclusively activates the tethered mammalian G protein but not the insect cell G proteins (Schneider et al. 2009). This was additionally supported by the lack of [^{35}S]GTPγS binding in membranes expressing non-fused hH_4R in the absence of mammalian G proteins (Schneider et al. 2009). The K_D value of [^{35}S]GTPγS in the presence of the full hH_4R agonist histamine or the inverse agonist thioperamide in membranes co-expressing hH_4R-$G\alpha_{i2}$ and $G\beta_1\gamma_2$ was significantly reduced as compared to the coexpression system, indicating enhanced efficiency of G protein activation (Schneider et al. 2009). A higher GTP affinity of $G\alpha_{i2}$ in the fusion protein was also reflected by a significantly decreased K_M value in the presence of histamine in steady-state GTPase assays. Moreover, a slight increase of constitutive activity in steady-state GTPase assays additionally demonstrates the increased efficiency of G protein activation in the fusion protein system (Schneider et al. 2009). Interestingly, the B_{max} value of the hH_4R-$G\alpha_{i2}$ fusion protein in immunoblots and [^{3}H]histamine binding assays was increased as compared to the non-fused receptor (Schneider et al. 2009). This suggests a chaperone-like stabilizing effect of $G\alpha_{i2}$, favoring membrane insertion of the receptor protein. Incubation of the cell culture with histamine or thioperamide did not further enhance the B_{max} value of the fusion protein in [^{3}H]histamine binding (Schneider et al. 2009), suggesting that the fusion of hH_4R to $G\alpha_{i2}$ induces already the maximum possible number of correctly folded receptors. An overview of the most important features of the hH_4R-$G\alpha_i$ fusion protein in comparison to the co-expression system (hH_4R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$) is provided in Table 4.

2.4.6 Role of Glycosylation for hH_4R Expression and Function

Western blotting of hH_4R-expressing Sf9 cell membranes revealed two bands at 43 and 46 kDa. Incubation of the baculovirus-infected Sf9 cell culture with the glycosylation inhibitor tunicamycin removed the 46 kDa band, indicating that this is most likely a glycosylated H_4R species (Schneider et al. 2009). Although the total protein amount on the Western blot was comparable for both untreated and tunicamycin-treated H_4R protein (2.5–3 pmol/mg as assessed by using FLAG-β_2AR standard membranes with known receptor expression levels), the B_{max} value in [^{3}H]histamine binding was reduced by 75% after tunicamycin treatment. Nevertheless, the K_D value of [^{3}H]histamine remained unchanged (Schneider et al. 2009). Thus, hH_4R deglycosylation does not significantly affect the [^{3}H]histamine binding site of functional hH_4R, although it significantly reduces the amount of correctly folded receptor protein.

The activation of $G\alpha_{i2}$ proteins by deglycosylated hH_4R was investigated in [^{35}S]GTPγS saturation binding and steady-state GTPase assays. Even in the presence of histamine, the deglycosylated hH_4R in the tunicamycin-treated membranes

activated $G\alpha_{i2}$ less efficiently than the glycosylated H_4R (increased K_D value of [^{35}S]GTPγS) (Schneider et al. 2009). Thus, proper glycosylation of hH_4R seems to be a prerequisite for efficient G protein coupling. By contrast, determination of the K_M value of GTP at the $G\alpha_{i2}$ subunit in steady-state GTPase assays only revealed a non-significant trend towards an increased K_M-value in the tunicamycin-treated membranes (Schneider et al. 2009).

In [^{35}S]GTPγS binding assays, deglycosylation reduced the constitutive activity of H_4R coexpressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ from 70 to 40% (Schneider et al. 2009). Neither the coupling factor from [^{35}S]GTPγS binding assays nor the turnover number from steady-state GTPase assays changed significantly, when hH_4R was deglycosylated (Schneider et al. 2009). This suggests that deglycosylation of hH_4R reduces efficacy of Gα activation without affecting the total number of activated G proteins.

2.4.7 Reasons for the High Constitutive Activity of hH_4R

The inactive state of GPCRs is established by intramolecular interactions that conformationally restrain the receptor. Data obtained from the rhodopsin molecule have led to the assumption that the so-called ionic lock is highly important for the inactivation of GPCRs (Palczewski et al. 2000; Vogel et al. 2008). The ionic lock is a salt bridge between a highly conserved glutamate in position 6.30 of TM6 and the arginine of the DRY motif located on the bottom of TM3 (position 3.50). The importance of the ionic lock for the regulation of odorant GPCR activity has been shown recently (de March et al. 2015). However, some receptors do not form an ionic lock, despite the presence of the required amino acids. This has been reported, e.g. for the human β_2AR (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007) or the human A_{2A} adenosine receptor (Jaakola et al. 2008), both of which show considerable constitutive activity.

The hH_4R is the only histamine receptor with an alanine in position 6.30, which precludes ionic lock formation (Schneider et al. 2010a) and possibly explains the observed high G protein-independent activity of the hH_4R (Schneider et al. 2009). To test this hypothesis, the TM6 part of the potential ionic lock was reconstituted by introducing the A6.30E mutation, and the resulting mutant was analyzed in the Sf9 cell expression system. Immunoblots and [^{3}H]histamine saturation binding indicated comparable expression levels of the mutant and the wild-type hH_4R. Unexpectedly, the pharmacological properties of hH_4R-A6.30E (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) in radioligand binding, steady-state GTPase assay and [^{35}S]GTPγS binding assays were basically unaltered as compared to the wild-type hH_4R (Schneider et al. 2010a). The replacement of alanine 6.30 by glutamate resulted in a slight but non-significant reduction of coupling factor ([^{35}S]GTPγS binding), turnover number (steady-state GTPase assay) and constitutive activity ([^{35}S]GTPγS binding and steady-state GTPase assay). This indicates that the ionic lock interaction was either not fully reconstituted or not sufficient to stabilize the inactive conformation of hH_4R (Schneider et al. 2010a). An overview of the most important features of the hH_4R-A6.30E mutation in comparison to the wild-type hH_4R is provided in Table 4.

Molecular modeling studies revealed potential interactions that may stabilize the active conformation despite the presence of the reconstituted ionic lock. The hH_4R active state was modeled in complex with the C terminus of $G\alpha_{i2}$ by using the crystal structures of the turkey β_1AR (Warne et al. 2008) and the human adenosine A_{2A} receptor (Jaakola et al. 2008) as templates. This revealed an additional salt bridge between D5.69 at the N-terminus of the second cytoplasmic loop (CL3) and R6.31, which may stabilize an active receptor conformation (Schneider et al. 2010a). Since D5.69 is nearly unique among the GPCRs for biogenic amines, this salt bridge may be at least partly responsible for the high constitutive activity of hH_4R and should be analyzed in future studies.

Recently, the reasons for the high constitutive activity of hH_4R were further elucidated (Wifling et al. 2015a, b). These studies made use of the large pharmacological differences between human and rodent H_4R (Schnell et al. 2011; Strasser et al. 2013). For example, constitutive activity of mH_4R and rH_4R is strongly reduced as compared to hH_4R (Schnell et al. 2011) and the inverse hH_4R agonist JNJ7777120 exhibits partial agonism at mH_4R and rH_4R. Moreover, the potency of the agonist histamine is lower for the rodent orthologues as compared to hH_4R (Schnell et al. 2011). Mutational studies indicate that position 169 of the second extracellular loop is an important determinant of the distinct agonist binding properties of human and mouse H_4R (Lim et al. 2008). The F169 of the hH_4R is replaced by a V169 in the mH_4R. Thus, Wifling et al. (2015b) performed a detailed analysis of the "mouse-like" hH_4R-F169V mutant in the Sf9 cell system. In fact, hH_4R-F169V exhibited decreased constitutive activity as compared to wild-type hH_4R, resulting in an increased agonistic effect of histamine. Moreover, histamine binding affinity as well as the inverse agonistic effect of thioperamide was reduced (Wifling et al. 2015b). The second key amino acid identified by Wifling et al. (2015b) was S179, which is replaced by methionine in the mH_4R and by alanine in the rH_4R. The double mutants hH_4R-F169V+S179A and hH_4R-F169V+S179M showed an even stronger reduction of constitutive activity as compared to the hH_4R-F169V single mutant (Wifling et al. 2015b). These results suggest that the constitutively active state of hH_4R at least partly depends on hydrophobic interactions between the extracellular domains of TM 5, 6, and 7 and ECL2. A hydrogen bond between S179 and T323 additionally stabilizes the agonist-free active state of the hH_4R (Wifling et al. 2015b).

These mutations, however, did not completely eliminate the constitutive activity of hH_4R. A total loss of constitutive activity was only achieved by introducing the F168A mutation (Wifling et al. 2015a). This indicates that – despite the strong reduction of constitutive activity in the hH_4R-F169V mutation – the adjacent amino acid in the FF motif, F168, is the key residue responsible for the high constitutive activity of hH_4R (Wifling et al. 2015a). An FF motif in ECL2 is also present in other GPCRs, e.g. β_2AR, hH_3R and M_2R, suggesting a similar role of the ECL2 conformation on constitutive activity of these receptors.

2.4.8 The Role of the DRY Motif in G Protein Activation by the Human hH_4R

The arginine R3.50 of the DRY motif at the bottom of TM3 stabilizes the inactive receptor state by forming a salt bridge with the adjacent D/E3.49 residue (Nygaard et al. 2009). Therefore, we analyzed the effect of the hH_4R-R3.50A mutation on constitutive activity and ligand binding in membranes co-expressing hH_4R-R3.50A, $G\alpha_{i2}$ and $G\beta_1\gamma_2$. Surprisingly, the R3.50A exchange totally eliminated G protein coupling as indicated by the complete absence of receptor-regulated steady-state GTPase activity (Schneider et al. 2010a). Moreover, the hH_4R-R3.50A mutant adopted an inactive state with reduced affinity of the agonist histamine and increased affinity of the inverse agonist thioperamide (Schneider et al. 2010a). However, introduction of the R3.50A mutation reduced histamine affinity only by 50% and did not affect B_{max}. This suggests that the hH_4R-R3.50A mutant still adopts a "residual" G protein-independent high-affinity state.

To explain the total loss of G protein coupling of the hH_4R-R3.50A mutant, molecular modelling studies were performed using the active-state of the hH_4R in complex with the C-terminus of $G\alpha_{i2}$. This analysis revealed that R3.50 of the hH_4R may interact with the backbone oxygens of C352 and G353 in the $G\alpha_{i2}$ C-terminus (Schneider et al. 2010a). This supports the adoption of the $G\alpha_{i2}$ conformation, which is required for interaction with TM6 of the receptor. Thus, the R3.50A mutation hampers G protein recognition by hH_4R. Nevertheless, the hH_4R-R3.50A mutant is still able to form the salt bridge between D5.69 and R6.31, which stabilizes an active state. This could explain why hH_4R-R3.50A still exhibits relatively high histamine affinity (Schneider et al. 2010a). However, the effect of mutations in the E/DRY motif is not disrupting G protein coupling in all GPCRs. Rovati et al. (2007) described two phenotypes P1 and P2 that are produced by mutations of the E/D3.49- or the R3.50-residue. While in P1-type receptors high-affinity agonist binding and G protein coupling are retained after mutating position R3.50, P2-type receptors show a disrupted receptor-G protein interaction and reduced agonist binding affinity (Rovati et al. 2007). Accordingly, the hH_4R belongs to the group of P2-type GPCRs. An overview of the most important features of the hH_4R-R3.50A mutation in comparison to the wild-type hH_4R is provided in Table 4.

2.4.9 Pharmacological Characterization of hH_4R Ligands

As explained above, co-expression of the hH_4R and its cognate mammalian G proteins in Sf9 cells results in high constitutive activity (Schneider et al. 2009). This reduces the maximum available signal range, yielding a very low signal-to-noise ratio. Even in the presence of 100 mM of NaCl, the full agonist histamine produced only a signal intensity of ~30% (related to baseline) (Schneider and Seifert 2009). The expression of an hH_4R-$G\alpha_{i2}$ fusion protein did not improve the signal-to-noise ratio, but resulted in even higher constitutive activity and reduced relative intensity of histamine-induced signals (Schneider et al. 2009). Thus, the properties of the hH_4R/G protein co-expression system and the hH_4R-$G\alpha_{i2}$ fusion protein are rather unfavorable for the characterization of hH_4R ligands.

This prompted us to perform a closer investigation of the effects of regulators of G protein signaling (RGS proteins). A common feature of RGS proteins is the 120 amino acid RGS domain, which interacts with Gα subunits and increases their intrinsic GTPase activity (Willars 2006). RGS proteins are classified in eight subfamilies that differ from each other by protein size and the presence of additional functional domains. They regulate the activity of $G\alpha_{i/o}$- or $G\alpha_q$ proteins, but no RGS protein-mediated activation of $G\alpha_s$ has been reported to date. Due to their mechanism of action, RGS proteins should enhance signal intensity in steady-state GTPase assays. In fact, fusion of the α_2AR C-terminus to the RGS4 N-terminus significantly increased α_2AR-mediated stimulation of GTPase activity (Bahia et al. 2003).

For the experiments with the hH_4R, the two RGS proteins RGS4 and GAIP (Gα-interacting protein; also known as RGS19) were selected. RGS4 and GAIP both exhibit a simple protein structure without additional functional domains. Therefore, only activation of $G\alpha_i$ GTPase activity is expected. Both RGS proteins were fused to the hH_4R via a His_6 linker (Fig. 5), very similar to the previously described hH_4R-$G\alpha_{i2}$ fusion protein approach (Fig. 3). The hH_4R-RGS fusion proteins were co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 cell membranes. The corresponding co-expression system was characterized by infecting Sf9 cells with baculoviruses encoding hH_4R, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4 or GAIP.

Both RGS4 and GAIP, irrespective of whether they were co-expressed or fused to hH_4R, increased the apparent K_M value of $G\alpha_{i2}$ in the presence of histamine in steady-state GTPase assays. This effect reached significance for the co-expressed GAIP and the hH_4R-RGS4 fusion protein (Schneider and Seifert 2009). By contrast, there was no effect of RGS proteins on the K_M value in the presence of the inverse agonist thioperamide (Schneider and Seifert 2009). This suggests that GPCR-mediated activation of the G protein is a prerequisite for the RGS protein effect.

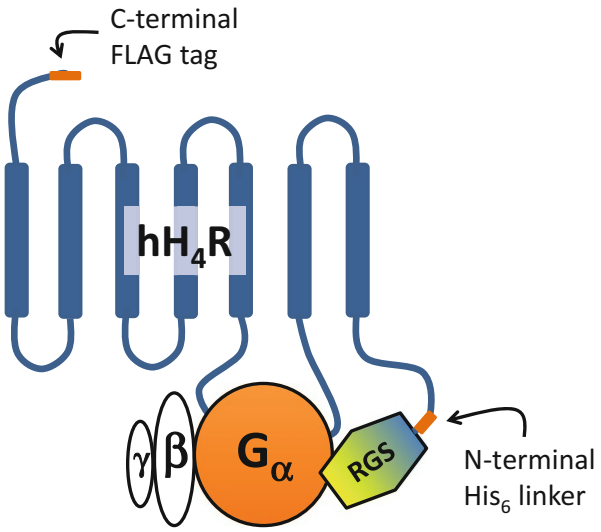

Fig. 5 Schematic depiction of the hH_4R-RGS fusion protein. The C-terminus of the hH_4R is fused to the N-terminus of the RGS protein by a hexahistidine linker. This brings the RGS protein into close proximity to the heterotrimeric G protein. Adapted from Schneider and Seifert (2010c)

Compared to the RGS4-free co-expression system ($hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$), both the quadruple expression system ($hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$ + RGS4) and the fusion protein system (hH_4R-RGS4 + $G\alpha_{i2} + G\beta_1\gamma_2$) yielded a significantly increased relative steady-state GTPase signal of the inverse agonist thioperamide, while the histamine-induced signal remained unaffected (Schneider and Seifert 2009). The only major difference between co-expressed and hH_4R-attached RGS4 was an increased baseline steady-state GTPase activity in the hH_4R-RGS4 fusion protein system, but an unaltered baseline, when RGS4 was co-expressed (Schneider and Seifert 2009).

Co-expression of GAIP with hH_4R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ had no significant effect on baseline activity or thioperamide- and histamine-induced signals in steady-state GTPase assays. However, when GAIP was fused to hH_4R, the histamine-induced relative signal in steady-state GTPase assays was significantly increased by ~69% and the thioperamide-induced signal was enhanced by ~45%. The baseline activity of the GAIP-hH_4R fusion protein system, however, remained unaffected (Schneider and Seifert 2009). Thus, in contrast to hH_4R-RGS4, the hH_4R-GAIP fusion protein (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) enhanced the absolute histamine-induced signal without changing baseline activity. Therefore, the relative stimulatory effect of histamine was increased (Schneider and Seifert 2009).

The different behavior of RGS4 and GAIP in the fusion proteins is surprising, because both RGS proteins have a similar RGS domain and no additional functionalities. Possibly, the differences are caused by distinct G protein affinities of these RGS proteins. According to the UniProtKB database entry P49795, GAIP binds to $G\alpha_i$ proteins in the rank order $G\alpha_{i3} > G\alpha_{i1} > G\alpha_o >> G\alpha_z/G\alpha_{i2}$. Thus, among the $G\alpha_i$ isoforms, $G\alpha_{i2}$ is the one with the lowest affinity to GAIP. This means that the effect of GAIP may only become visible, when the number of activated $G\alpha_{i2}$ subunits exceeds a certain threshold. While under basal conditions the number of activated $G\alpha_{i2}$ subunits is too low for a visible hH_4R-GAIP-mediated effect, stimulation by histamine increases the number of active $G\alpha_{i2}$ to a level, where the GAIP-mediated effect becomes visible. By contrast, RGS4 may exhibit a higher $G\alpha_{i2}$ affinity than GAIP and therefore show already an effect under basal conditions. This hypothesis, however, should be tested by a side-by-side comparison of the $G\alpha_{i2}$ protein affinity of RGS4 and GAIP.

Co-expression of the hH_4R-GAIP fusion protein with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ produces a system with improved signal-to-noise ratio as compared to the standard co-expression system ($hH_4R + G\alpha_{i2} + G\beta_1\gamma_2$). A comparison of hH_4R-GAIP and wild-type hH_4R (both co-expressed $G\alpha_{i2}$ and $G\beta_1\gamma_2$) in steady-state GTPase assays revealed comparable pharmacological properties. First, potency and efficacy of selected hH_4R standard ligands were unaltered. Second, similar to wild-type hH_4R, the hH_4R-GAIP fusion protein exhibited sodium chloride-insensitive constitutive activity (Schneider and Seifert 2009). Third, the hH_4R-GAIP fusion protein showed an unchanged G protein selectivity profile as compared to the unmodified hH_4R protein (Schneider et al. 2009; Schneider and Seifert 2009). The unaltered G protein profile is surprising, because GAIP shows distinct affinities to different $G\alpha_i$ isoforms, which should theoretically influence the interaction between hH_4R-GAIP

and the G protein. The results, however, indicate that the G-protein-specificity of the hH_4R-GAIP fusion protein is governed by the properties of the receptor rather than by the RGS protein part. In summary, the hH_4R-GAIP fusion protein (co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$) can fully replace the standard co-expression system (hH_4R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$) in steady-state GTPase assays and allows the functional characterization of new hH_4R ligands with higher sensitivity and signal-to-noise ratio. The hH_4R-GAIP fusion protein approach was successfully used to evaluate a new class of N^G-acylated imidazolylpropyl-guanidine-derived hH_4R agonists (Ghorai et al. 2008; Igel et al. 2009b) or of cyanoguanidine-related hH_4R agonists (Igel et al. 2009a; Geyer et al. 2016). An overview of the most important features of the various H_4R/RGS fusion protein and co-expression approaches in comparison to the "standard" co-expression system (hH_4R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$) is provided in Table 4.

2.4.10 Structure-Activity Relationships of hH_4R Inverse Agonists

The high constitutive activity of hH_4R significantly reduces the signal-to-noise ratio in steady-state GTPase assays and reduces the sensitivity of agonist assays. However, this feature becomes an advantage, when inverse agonists are characterized. The hH_4R may maintain its constitutive activity under physiological conditions, because it is resistant to high sodium concentrations. As hypothesized by Schneider et al. (2009), on the one side, inverse agonists could be therapeutically advantageous in case of pathophysiologically increased constitutive H_4R activity, because they may exert a stronger anti-pruritic effect than neutral antagonists. On the other side, the re-folding of misfolded H_4R protein observed with the inverse agonist thioperamide (Schneider et al. 2009) (Sect. 2.4.4) may be a general effect of inverse H_4R agonists. Thus, inverse agonist-mediated upregulation of intact H_4R protein may result in rebound effects after drug discontinuation (Schneider et al. 2009). Although these hypotheses were not proven yet under physiological conditions, they illustrate the potential importance of characterizing inverse H_4R agonism during drug development. Therefore, structure-activity relationships for hH_4R inverse agonism should be established.

A series of 25 previously described (Venable et al. 2005) H_4R ligands (indoles, benzimidazoles, and thienopyrroles; Fig. 6) structurally derived from the prototypical H_4R antagonist JNJ7777120 (Thurmond et al. 2004) was characterized in [^{3}H]histamine binding assays and steady-state GTPase assays using membranes expressing hH_4R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$. The steady-state GTPase assays were performed in the absence of sodium chloride to obtain maximum constitutive activity.

The steady-state GTPase assay data reveal that most of the compounds were inverse agonists with a lower efficacy than thioperamide. Only three of the 25 compounds (~12%) were neutral antagonists (Schneider et al. 2010b). This confirms a previous analysis of literature data on 380 antagonists binding to 73 GPCRs. Only 15% of these compounds were neutral antagonists (Kenakin 2004). Thus, neutral antagonism seems to be a rare phenomenon.

In general, the pK_b values from steady-state GTPase assays in the presence of histamine fit very well to the pK_i values from [^{3}H]histamine binding. In a subset of

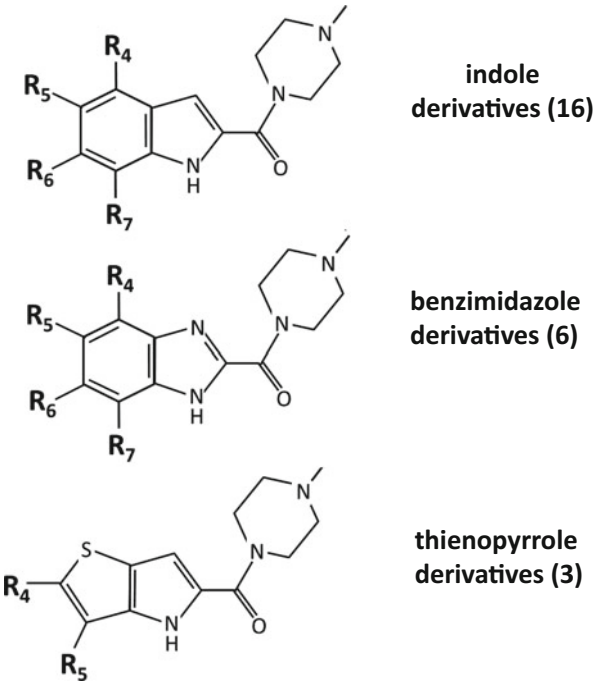

Fig. 6 Scaffold structure of three classes of H_4R antagonists/inverse agonists. The *numbers* in *brackets* indicate the number of compounds tested

compounds, the pEC_{50} values determined in the absence of histamine were significantly lower than the pK_i and/or pK_b values. Such discrepancies have been reported before for inverse agonists, e.g. at the hH_4R (Smits et al. 2008) or the β_2AR (Chidiac et al. 1994). Maybe, this subset of hH_4R antagonists discriminates between the agonist-free constitutively active receptor and the histamine-activated receptor state (Schneider et al. 2010b). These observations confirm the insufficiency of the two-state model of receptor activation and point to the existence of ligand-specific receptor states.

The potential binding mode of inverse hH_4R agonists of the indole series was analyzed by molecular dynamics simulations with the completely unsubstituted indole compounds (R4-7 = H; Fig. 6). The positively charged piperazine amino group interacts electrostatically with the highly conserved $Asp^{3.32}$. Moreover, both the carbonyl moiety and the indole NH of the ligand establish an interaction with the side chain of the uncharged $Glu^{5.46}$. The indole moiety of the ligand shows a hydrophobic interaction with the indole part of $Trp^{6.48}$ (Schneider et al. 2010b). $Trp^{6.48}$ is a key player in the so-called rotamer toggle switch mechanism of receptor activation, which had been previously postulated for the β_2AR (Shi et al. 2002). The stabilization of $Trp^{6.48}$ in its vertical conformation by the indole-derived ligand is a typical feature of the inactive receptor conformation and may explain the inverse agonism of such compounds. The benzimidazole-related structures bind in a similar way, but, in contrast to the indole-derived compounds, they form two tautomers with distinct binding modes (Schneider et al. 2010b).

Replacement of the R5/R7 hydrogen of the indole derivatives by the more space-filling chlorine increases H_4R binding affinity. Molecular dynamics simulations suggest that two small binding pockets in the H_4R protein may be filled by these chlorine residues, which increases the ligand-receptor contact area (Schneider et al. 2010b). Substitution of R5 by -OCH_3 reduces binding affinity, suggesting that larger substituents may be unfavorable. However, there is no significant correlation between molar volume and affinity of a series of indole compounds, suggesting that the volume of R5 may not be the only descriptor that influences binding affinity (Schneider et al. 2010b). By contrast, the size of R5 correlates excellently with the inverse agonistic efficacy of a subset of eight indole-derived compounds with varying R5 substituents. A calculation of the descriptors logP, molar refractivity, molar volume, polarizability, refraction index and polar surface area revealed that inverse agonistic efficacy solely depended on molar volume, but not on the other factors. The inverse agonistic efficacy of these compounds was inversely correlated to the molar volume of the substituent R5 (Schneider et al. 2010b).

In summary, despite the limited number of compounds and substitution patterns available, in this study the first structure-activity relationships for inverse H_4R agonism were identified. It was, however, not possible to predict all changes in binding mode and receptor conformation that result from small structural alterations of the ligand. Moreover, a general model that applies to structurally distinct classes of hH_4R inverse agonists could not be established yet. In the future, the hH_4R should be co-crystallized with various inverse agonists to elucidate the exact binding mode of these compounds. Although this would be a very ambitious project, the numerous crystallized ligand-receptor complexes published in the recent years (Cherezov et al. 2007; Rasmussen et al. 2007, 2011; Shonberg et al. 2015) demonstrate that this is not impossible.

3 Summary and Outlook

In this chapter, the results from the characterization of all four histamine receptor subtypes in the Sf9 insect cell system were summarized. On the one hand, it might be argued that insect cells do not represent physiological conditions as well as primary cells. On the other hand, it is difficult to isolate primary cells in sufficiently high numbers. Moreover, a side-by-side comparison of receptor isoforms or species orthologues in a defined environment is virtually impossible in primary cells. Since cells from different tissues have to be used, cell type-specific properties like crosstalk with other receptors or special features of the signaling pathways can lead to heterogeneous results, even for the same receptor isoform. Also, for some receptors like H_3R, no suitable primary cell system is available (Seifert et al. 2013).

Thus, for a comparison of the intrinsic properties of GPCR isoforms, e.g. G protein affinity/selectivity or constitutive activity, Sf9 cells represent a superior option. As explained in this chapter, Sf9 cells do not contain background GPCR activity and do not produce endogenous agonists activating mammalian GPCRs. Moreover, Sf9 cells allow the co-expression of defined mammalian $G\alpha_s$ or $G\alpha_i$

protein subunits on a "clean" signaling background. This was demonstrated by the analysis of the hH_2R interaction with long and short $G\alpha_s$ splice variants or by in-depth studies of hH_3R/hH_4R $G\alpha_i$ isoform specificity and ion sensitivity. Table 5 shows numerous aspects of histamine receptor pharmacology addressed by using the Sf9 insect cell expression system.

The ligand binding studies and the G protein activation assays discussed in this chapter were all performed with radiolabeled reagents. Radioactivity-based assays, however, are increasingly hampered by legal overregulation and growing waste disposal costs. In this situation, fluorescence-based GPCR ligand binding and G protein activation assays could represent interesting alternatives. Unfortunately, many histamine receptor ligands are rather small molecules and easily lose binding affinity when coupled to a bulky fluorophore. Nevertheless, some progress has been made during the past years. For example, a cyanine dye-labeled aminopotentidine derivative exhibited nanomolar hH_2R potency (Xie et al. 2006c). Moreover, fluorescent pyrylium- or cyanine-labeled dimeric carbamoylguanidines were synthesized, but these compounds failed in binding assays due to intracellular accumulation and the resulting high fluorescence background (Kagermeier et al. 2015). A high-affinity fluorescent H_1R antagonist was obtained by labeling mepyramine with a BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-derived dye (Rose et al. 2012). Fluorescent hH_3R-selective ligands were developed by using the chalcone partial structure (Tomasch et al. 2012). Moreover, a compound named "Bodilisant," which has been reported recently, is a BODIPY-labeled non-imidazole ligand with nanomolar hH_3R affinity (Tomasch et al. 2013). Some progress has also been made in the field of fluorescence-based G protein activation assays. For example, a europium-labeled non-hydrolysable GDP derivative can replace [^{35}S]GTPγS in GTPγS binding assays (Koval et al. 2010). This enables a time-resolved fluorescence-based assay that is, e.g., suited for the functional characterization of hH_3R ligands (Singh et al. 2012).

The functional assays described in this chapter focused on the determination of GPCR-mediated G protein activation (steady-state GTPase and [^{35}S]GTPγS binding assays). However, GPCRs can additionally activate G protein-independent signaling mechanisms, most importantly through β-arrestin recruitment (Lefkowitz and Shenoy 2005; Shukla et al. 2014). The hH_4R ligand JNJ-7777120, which acts as an inverse hH_4R agonist in G protein activation assays (Schneider et al. 2009), unexpectedly turned out to be an agonist with regard to hH_4R-mediated β-arrestin recruitment (Rosethorne and Charlton 2011). This phenomenon is also known as "biased signaling" or "functional selectivity" and has important implications for drug development (Seifert et al. 2011; Nijmeijer et al. 2013). In future studies, biased signaling of hH_1R, hH_2R, or hH_3R and functional selectivity of the corresponding ligands should be investigated in more detail.

However, the most important, but also most ambitious, goal in future studies would be the crystallization of all four histamine receptor subtypes. To date, only the crystal structure of the hH_1R has been resolved (Shimamura et al. 2011). The crystal structures of the histamine receptors are required to answer several still unresolved questions. For example, exact knowledge of the hH_4R conformation could help to explain, why this receptor shows such a high constitutive activity

Table 5 Various aspects of histamine receptor pharmacology and medicinal chemistry investigated in the Sf9 insect cell expression system

Aspect	Receptor subtype	Example	Reference
Species-specificity of receptor pharmacology	H_1R	• Efficacy/potency of some agonistic bulky 2-phenylhistamines and histaprodifens: $gpH_1R > hH_1R$ • Potency of several arpromidine-type H_1R antagonists: $gpH_1R > hH_1R$	Seifert et al. (2003)
	H_2R	• Affinity of large guanidine-type agonists in [^{3}H]tiotidine binding: $hH_2R\text{-}Gs\alpha_S < gpH_2R\text{-}Gs\alpha_S$ • GTPγS-sensitivity of high-affinity agonist binding: $hH_2R\text{-}Gs\alpha_S > gpH_2R\text{-}Gs\alpha_S$ • potencies and efficacies of guanidines (steady-state GTPase): $gpH_2R\text{-}Gs\alpha_S > hH_2R\text{-}Gs\alpha_S$	Kelley et al. (2001)
	H_3R	Imoproxifan: nearly full agonist at hH_3R, but inverse agonist at rH_3R (steady-state GTPase)	Schnell et al. (2010b)
	H_4R	Constitutive activity of mH_4R and $rH_4R < hH_4R$; inverse hH_4R agonist JNJ7777120 is a partial agonist at cH_4R, mH_4R and rH_4R; histamine potency at cH_4R, mH_4R and $rH_4R < hH_4R$	Schnell et al. (2011)
Studies with chimeric receptor proteins	H_1R	N-terminus and ECL2 of hH_1R replaced by guinea-pig sequences ($h(gpNgpE2)H_1R$): higher maximum G_q-activation and lower histamine potency as compared to hH_1R or $h(gpE2)H_1R$; extracellular surface of the H_1R influences ligand binding, recognition and guiding into the binding pocket	Strasser et al. (2008b) and Wittmann et al. (2011)
	H_2R	Comparison of $hH_2R\text{-}A271D\text{-}Gs\alpha_S$, $NhCgpH_2R\text{-}Gs\alpha_S$, $NgpChH_2R\text{-}Gs\alpha_S$, $hH_2R\text{-}Gs\alpha_S$ and $gpH_2R\text{-}Gs\alpha_S$ to investigate the causes for the pharmacological differences between hH_2R and gpH_2R with regard to large guanidine-type agonists	Kelley et al. (2001)

(continued)

Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
Importance of individual amino acids for defined receptor functions	H_1R	hH_1R-F153L and hH_1R-I433V: reduced expression, function and [^{3}H]mepyramine affinity, altered electrophoretic mobility; hH_1R-F153L/I433V double mutant: excellent expression, but only partial change of pharmacological properties (compared to wild-type hH_1R)	Seifert et al. (2003)
	H_2R	hH_2R-K173A-Gsα_S or hH_2R-K175A-Gsα_S: reduced histamine-induced steady-state GTPase signals	Preuss et al. (2007c)
	H_3R	hH_3R-D2.50N: interaction with Gα_{i3} disrupted, but still activation of Gα_{i1}, Gα_{i2} and Gα_{o1}	Schnell and Seifert (2010)
	H_4R	hH_4R-F168A: Contribution of ECL2 to ligand binding and constitutive activity	Wifling et al. (2015a)
		hH_4R-R3.50A: DRY motif important for G protein coupling	Schneider et al. (2010a)
Medicinal chemistry, SARs for ligands	H_2R	Replacement of the imidazolyl moiety in the imidazolylpropylguanidine structure by an aminothiazol moiety increases selectivity for H_2R over H_1R, H_3R and H_4R	Kraus et al. (2009)
	H_4R	N^G-acylation increases hH_4R affinity and efficacy of the partial H_3R/H_4R agonist 3-(1*H*-Imidazol-4-yl)propylguanidine (SK&F 91486) and reduces efficacy at hH_3R → increase of hH_4R selectivity	Igel et al. (2009b)
		5-Methyl substitution of imbutamine increases selectivity for hH_4R over hH_3R	Geyer et al. (2014)
Factors reducing constitutive activity	H_1R	Weak inverse agonism of some antidepressants or antipsychotics (e.g., chlorpromazine)	Appl et al. (2012)
	H_2R	hH_2R-Gsα_S or hH_2R-Gsα_L: weak inverse agonism of ranitidine and famotidine (efficacy higher at hH_2R-Gsα_L than at hH_2R-Gsα_S)	Wenzel-Seifert et al. (2001)
	H_3R	Inverse agonist: thioperamide; inhibition of hH_3R constitutive activity by anions or cations:	Schnell and Seifert (2010)

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Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
		$Li^+ \sim Na^+ \sim K^+ < Cl^- < Br^- < I^-$; strongest effect of sodium in the presence of $G\alpha_{i3}$	
	H_4R	(Partial) inverse agonist: thioperamide; insensitivity of constitutive activity to sodium	Schneider et al. (2009)
Stoichiometry of receptor/G protein coupling	H_3R	Coupling ratio in co-expression system: 1:2 ($G\alpha_{i1}$), 1:6 ($G\alpha_{i2}$); 1:3 ($G\alpha_{i3}$); 1:11 ($G\alpha_{o1}$)	Schnell et al. (2010a)
	H_4R	Coupling ratio in $hH_4R/G\alpha_{i2}$ co-expression system: ~1:5; hH_4R-$G\alpha_{i2}$ fusion protein: ~1:1	Schneider et al. (2009)
Selectivity for closely related G proteins	H_2R	hH_2R-Gsα fusion protein: similar apparent constitutive activity with $Gs\alpha_L$ and $Gs\alpha_S$	Wenzel-Seifert et al. (2001)
	H_3R	Interaction of hH_3R with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{io1}$ (co-expression): no pharmacological differences	Schnell et al. (2010a)
	H_4R	Co-expression system, comparison of hH_4R coupling to $G\alpha_{i1\text{-}3}$ and $G\alpha_o$: most efficient activation of $G\alpha_{i2}$, least efficient interaction with $G\alpha_o$	Schneider et al. (2009)
Role of N-terminal glycosylation for receptor function	H_4R	Tunicamycin (glycosylation inhibitor): no effect on total hH_4R protein expression and on [^{3}H]histamine affinity, but reduction of B_{max} value → N-terminal glycosylation important for correct folding; less efficient activation of $G\alpha_{i2}$ by deglycosylated hH_4R	Schneider et al. (2009)
Function of RGS proteins in receptor/G protein coupling	H_1R	Co-expression of hH_1R with RGS4 or GAIP unmasks a productive interaction with insect cell $G\alpha_q$	Houston et al. (2002)
	H_4R	hH_4R-RGS4 fusion protein: increase of baseline steady-state GTPase activity and of thioperamide inverse agonistic effect (compared to hH_4R); hH_4R-GAIP: pharmacological properties unchanged (compared to hH_4R), but significantly increased relative histamine- and thioperamide-induced GTPase signals	Schneider and Seifert (2009)

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Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
Analysis of the G protein cycle	H_2R	Different **ternary complex stabilization** of hH_2R-$G\alpha_s$ or gpH_2R-$G\alpha_s$ by N^G-acylated imidazolylpropylguanidines (AIPGs) as compared to the corresponding guanidines	Xie et al. (2006a)
	H_4R	**GTPγS binding**: GTPγS affinity of $G\alpha_{i2}$ (co-expressed with hH_4R and $G\beta_1\gamma_2$) was significantly reduced by the inverse agonist thioperamide; fusion of $G\alpha_{i2}$ to hH_4R increases $G\alpha_{i2}$ affinity	Schneider et al. (2009)
	H_4R	**GTP hydrolysis**: a GTP turnover number of ~4 was determined for hH_4R (+$G\beta_1\gamma_2$, +$G\alpha_{i2}$); the influence of regulators of G protein signaling (co-expressed or fused to hH_4R) on the K_M value of the $G\alpha_{i2}$ GTPase activity was determined	Schneider et al. (2009) and Schneider and Seifert (2009)
	H_2R	**Effector activation**: Comparison of AC activity (basal, with GTP and with GTP/histamine) in membranes expressing h, gp, r or cH_2R (alone or combined with $Gs\alpha_s$ or fused to $Gs\alpha_s$) → highest basal and GTP-induced AC activity detected for cH_2R	Preuss et al. (2007a)
Probing of receptor models	H_4R	**Two-state model**: high tendency of the hH_4R to occur in the active state; no stabilization of the inactive state by sodium; equilibrium shifted to the inactive side by thioperamide and other inverse agonists	Schneider et al. (2009)
	H_3R, H_4R	**Protean agonism**: Steady-state GTPase assays with membranes co-expressing hH_3R with $G\beta_1\gamma_2$ plus $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_{o1}$ as well as with membranes co-expressing hH_3R-$G\alpha_{i2}$ or hH_3R-$G\alpha_{o1}$ did not confirm the previously reported protean agonism of proxyfan	Schnell et al. (2010a)
		Comparison of H_4R mutants/species orthologues with different constitutive activities: JNJ-7777120 is a protean agonist	Wifling et al. (2015b)

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Table 5 (continued)

Aspect	Receptor subtype	Example	Reference
	H_1R, H_2R	**Ligand-specific conformations**: hH_2R, gpH_2R or rH_2R: AIPGs stabilize different active conformations as compared to guanidines. hH_1R: Unlike guanidines, AIPGs stabilize a partially active state	Xie et al. (2006a)
Comparison of receptors coupled to the same G protein type	β_2AR/ hH_2R ($G\alpha_s$)	Higher constitutive activity of β_2AR-$Gs\alpha_L$ as compared to β_2AR-$Gs\alpha_s$, but comparable constitutive activity of hH_2R-$Gs\alpha_L$ and hH_2R-$Gs\alpha_s$ → GDP affinity of G proteins does not influence the constitutive activity of all GPCRs	**β_2AR**: Seifert et al. (1998b) **hH_2R**: Wenzel-Seifert et al. (2001)
	hH_3R/ hH_4R ($G\alpha_{i2}$)	Steady-state GTPase assays: unlike hH_4R, the hH_3R exhibits NaCl-sensitive constitutive activity	**hH_3R**: Schnell and Seifert (2010) **hH_4R**: Schneider et al. (2009)
	hH_4R/ FPR1 ($G\alpha_{i2}$)	hH_4R: high NaCl-resistant constitutive activity, no effect of GTPγS on high-affinity agonist ([^{3}H]histamine) binding; FPR1: high NaCl-sensitive constitutive activity, dramatic reduction of high-affinity agonist ([^{3}H]fMLF) binding by GTPγS	**hH_4R**: Schneider et al. (2009) **FPR1**: Wenzel-Seifert et al. (1998)

(Schneider et al. 2009; Wifling et al. 2015a). Moreover, a crystal structure of the hH_3R may provide important information about the hH_3R-G protein interaction interface and possibly answer the question, why the hH_3R discriminates between $G\alpha_{i3}$ and other $G\alpha_{i/o}$ isoforms (Schnell and Seifert 2010). Furthermore, an hH_3R crystal may lead to the identification of the anion binding sites responsible for the monovalent anion-mediated reduction of constitutive hH_3R activity (Schnell and Seifert 2010). Finally, the knowledge of H_xR crystal structures could lead to the development of compounds that alter H_xR function as allosteric modulators. The concept of GPCR modulation by allosteric ligands is well established, and such ligands have been identified, e.g. for dopamine, muscarinic, adenosine, or chemokine receptors (Christopoulos 2014). By contrast, to the best of our knowledge, to date nothing is known about allosteric modulation of histamine receptors.

As a prerequisite for the preparation of H_xR crystals, high amounts of receptor protein have to be expressed, e.g. in Sf9 cells. After purification and solubilization,

the physical properties of the receptors can be investigated, e.g. with fluorescence-based methods. Such studies have been previously performed with the β_2AR (Gether et al. 1995; Kobilka 1995; Neumann et al. 2002) and were important steps towards the final goal of receptor crystallization (Cherezov et al. 2007; Rasmussen et al. 2007, 2011).

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Histamine Release from Mast Cells and Basophils

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Contents

Abstract

Mast cells and basophils represent the most relevant source of histamine in the immune system. Histamine is stored in cytoplasmic granules along with other

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amines (e.g., serotonin), proteases, proteoglycans, cytokines/chemokines, and angiogenic factors and rapidly released upon triggering with a variety of stimuli. Moreover, mast cell and basophil histamine release is regulated by several activating and inhibitory receptors. The engagement of different receptors can trigger different modalities of histamine release and degranulation. Histamine released from mast cells and basophils exerts its biological activities by activating four G protein-coupled receptors, namely H1R, H2R, H3R (expressed mainly in the brain), and the recently identified H4R. While H1R and H2R activation accounts mainly for some mast cell- and basophil-mediated allergic disorders, the selective expression of H4R on immune cells is uncovering new roles for histamine (possibly derived from mast cells and basophils) in allergic, inflammatory, and autoimmune disorders. Thus, the in-depth knowledge of mast cell and basophil histamine release and its biologic effects is poised to uncover new therapeutic avenues for a wide spectrum of disorders.

Keywords
Basophil • Degranulation • Histamine • Histamine receptors • Mast cell

1 Introduction

Mast cells and basophils are the major source of the biogenic amine histamine among immune cells (Graham et al. 1955; Riley and West 1952, 1953). These cells store histamine in cytoplasmic granules along with other amines, proteases, proteoglycans, some cytokines/chemokines, and angiogenic factors that are rapidly released upon triggering with a variety of stimuli (Stone et al. 2010; de Paulis et al. 2006; Marone et al. 2005; Detoraki et al. 2009). Although several differences exist between mast cells and basophils, the stimuli that induce their activation and the mechanisms of degranulation, histamine release from mast cells and basophils is regarded as a central event in the development of rapid anaphylactic reactions and allergic responses due to its activation of histamine receptors expressed on vascular and stromal cells as well as immune cells. In this chapter we will introduce the biology of mast cells, basophils, histamine and histamine receptors to review recent advancements in mast cell and basophil degranulation and histamine release.

2 Biology of Mast Cells and Basophils

Mast cells and basophils are characterized by the expression of the tetrameric ($\alpha\beta\gamma_2$) high affinity receptor for IgE (FcεRI) and the ability to synthesize histamine (Stone et al. 2010; Marone et al. 2005). Nevertheless, they also show crucial differences. Basophils are a rare population of fully mature, short-lived circulating immune cells (they account for approximately 1% of blood leukocytes) that are recruited to tissues upon inflammation (Karasuyama et al. 2011; Borriello et al. 2014a; Marone et al. 2014). On the other hand, mature mast cells are tissue-resident cells distributed throughout mucosal and connective tissues, often in close proximity

to blood and lymphatic vessels, near or within nerves, and beneath epithelial surfaces (Galli and Tsai 2012). In response to IgE crosslinking (e.g., antigens, superantigens, anti-IgE) or IgE-independent stimuli (e.g., cytokines, anaphylatoxins, proteases, Toll-like receptor [TLR] ligands) mast cells and basophils release a partially overlapping set of preformed (e.g., histamine, proteases, some cytokines) and de novo synthesized (e.g., lipids, cytokines/chemokines, angiogenic and lymphangiogenic factors) mediators, albeit differences exist (Stone et al. 2010; de Paulis et al. 2006; Marone et al. 2005; Detoraki et al. 2009; Galli and Tsai 2012; Borriello et al. 2014b; Voehringer 2012; Moon et al. 2014; Patella et al. 2000; Genovese et al. 2003). For example, prostaglandin D_2 (PGD_2) is synthesized only by mast cells, while interleukin (IL)-4 (an important cytokine for the development of type 2 immunity) is produced mainly by basophils.

Mast cells and basophils play a major role in the development of anaphylactic reactions and allergic responses (Stone et al. 2010; Marone et al. 2005). However, their involvement has also been shown in several pathophysiological conditions, such as acute and chronic response to pathogens (including but not limited to ticks and other ectoparasites) (Chan et al. 2012; Eberle and Voehringer 2016), cancer development and progression (Varricchi et al. 2016; Marichal et al. 2013a; Melillo et al. 2010; Visciano et al. 2015), and also resistance to animal venoms (Akahoshi et al. 2011; Metz et al. 2006; Schneider et al. 2007). In this regard, mast cells enhance innate resistance of mice to venoms derived from several species of snakes, the venomous lizard Gila monster, scorpions, and the honeybee at least in part by releasing proteases (i.e., carboxypeptidase A3 and chymase MCP4) that degrades toxins present in some of these venoms. Moreover, mast cell activation by IgE specific for components of honeybee venom or Russell's viper venom protects mice from challenge with lethal doses of these venoms (Marichal et al. 2013b). Interestingly, basophils also exert non-redundant roles in some experimental models (Karasuyama et al. 2011). For example, basophils are required for acquired resistance against *Haemaphysalis longicornis* second infestation (Wada et al. 2010).

In conclusion, the release of mast cell and basophil mediators (including histamine) is involved in several pathophysiological conditions and may result in either beneficial or detrimental effects.

3 Histamine and Histamine Receptors

The first physiological characterization of β-imidazolylethylamine (the chemical formula of histamine) was reported in 1910 by Dale and Laidlaw (Dale and Laidlaw 1910). They demonstrated that this molecule causes vasodilation, the contraction of smooth muscles in the airway, uterus, and the intestine, stimulates heart rate and contractility, and induces a shock-like syndrome when injected into animals. Further investigations showed that histamine also stimulates stomach hydrochloric acid secretion (Popielski 1920). Moreover, in 1924 Lewis and Grant described the classic "Triple Response" elicited by the subcutaneous injection of histamine: a red

spot due to vasodilatation, a wheal due to increased permeability, and flare due to an axon reflex (Lewis and Grant 1924). Nevertheless, the first demonstration of the physiological relevance of histamine came in 1927 when histamine was isolated from liver and lungs (Best et al. 1927). Later, histamine was also recognized as a mediator of experimental anaphylaxis (Feldberg and Kellaway 1937; Feldberg and Keogh 1937; Feldberg and O'Connor 1937). Of note, Riley and West (1952, 1953) demonstrated that mast cells are the predominant cellular source of histamine (Riley and West 1952, 1953). Subsequently, basophils were identified as the main source of histamine among blood cells (Graham et al. 1955).

Histamine binds to four G protein-coupled receptors (GPCRs), namely H1-receptor (H1R), H2-receptor (H2R), H3-receptor (H3R), H4-receptor (H4R) (Panula et al. 2015; Seifert et al. 2013) (Fig. 1). H1R activation mediates many symptoms of type I allergic reactions, including pruritus, erythema and edema. Indeed, H1R

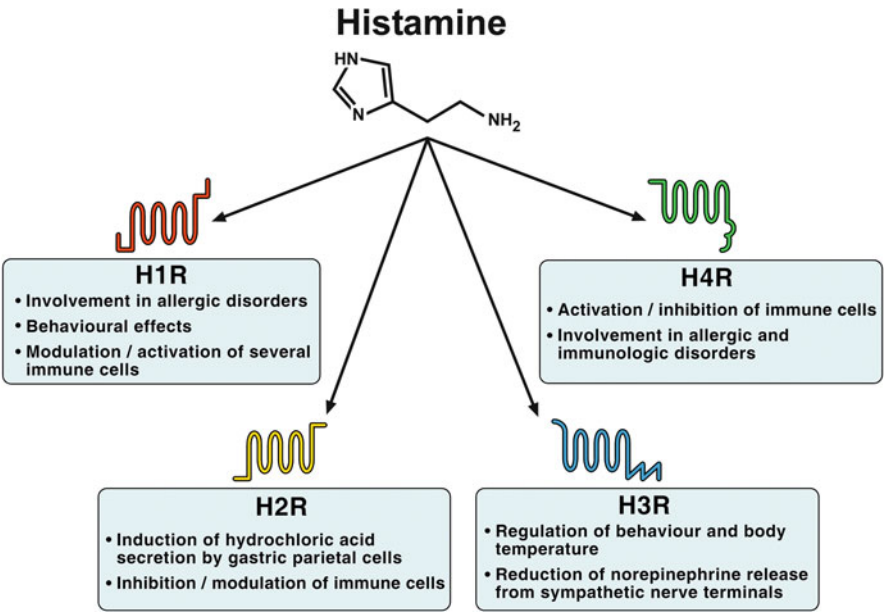

Fig. 1 Biological effects of histamine. Histamine exerts its effects through the engagement of four G-protein coupled receptors (H1R, H2R, H3R, H4R). H1R is expressed on endothelial cells and bronchial smooth muscle cells and plays a major role in allergic disorders. The presence of H1R in the central nervous system mediates several behavioral effects. H1R activation also exerts proinflammatory and immunomodulatory activities due to its expression on several immune cells (e.g., dendritic cells, macrophages, Th1 cells). H2R activation induces the secretion of hydrochloric acid from gastric parietal cells and modulates/inhibits a variety of immune cells (e.g., mast cells, basophils, neutrophils, eosinophils, dendritic cells, γδ cells, Th1 and Th2 cells). H3R regulates various aspects of behavior and body temperature at the level of central nervous system. In addition, H3R inhibits norepinephrine release from sympathetic nerve terminals in the heart. H4R modulates the migration and activation of a wide spectrum of immune cells (e.g., mast cells, basophils, eosinophils, monocytes, dendritic cells, NK, iNK T and γδ cells, $CD8^+$ T cells, Treg and Th2 cells) and is thereby involved in allergic and immune-mediated disorders

antagonists are used for local and systemic treatment of these symptoms (although H2R is also involved in the pathophysiology of IgE-mediated systemic anaphylaxis) (O'Mahony et al. 2011; Wechsler et al. 2013). In addition, H1R knockout mice have impairment in locomotor activity and exploratory behavior (Inoue et al. 1996), a decrease in aggression and anxiety (Yanai et al. 1998), a significant impairment in nociception and an enhancement in the sensitivity to the analgesic effect of morphine (Mobarakeh et al. 2000, 2002). H1R knockout mice also show an impairment of the immune response since several immune cell subsets express H1R. H1R deletion results in lower percentages of IFN-γ-producing T cells and more ovalbumin (OVA)-specific IgG1 and IgE compared with wild-type (Jutel et al. 2001). Interestingly, although allergen-stimulated T cells from H1R knockout mice exhibit an enhanced production of Th2 cytokines, allergen-challenged H1R knockout mice show reduced lung Th2 cytokines associated with lower airway inflammation, goblet cell metaplasia, and airway hyperresponsiveness. These conflicting results can be explained, at least in part, by considering that histamine promotes T cell chemotaxis. Thus, defective T cell trafficking could be responsible for reduced lung inflammation in allergen-challenged H1R knockout mice (Bryce et al. 2006). In addition to T cells, human lung macrophages, monocyte-derived macrophages, and monocyte-derived dendritic cells express higher levels of H1R compared with precursor monocytes. Histamine induces the release of proinflammatory mediators (β-glucuronidase, IL-8 and IL-6) by MDM and HLM through the activation of H1R (Triggiani et al. 2001, 2007; Marone et al. 2001).

H2R is expressed on the parietal cells of the stomach and its activation induces hydrochloric acid secretion. Nevertheless, H2R knockout mice are phenotypically normal and show normal basal gastric pH due to gastric mucosa hypertrophy and increased circulating levels of gastrin (Kobayashi et al. 2000). Interestingly, these mice show a dysregulated T lymphocyte activity, that is upregulation of both Th1 and Th2 cytokines and decreased OVA-specific IgE production compared with wild-type and H1R knockout mice (Jutel et al. 2001). H2R is extensively expressed among immune cells. H2R gene expression increases in human IL-4^+ T cells upon bee venom exposure of non-allergic beekeepers (Meiler et al. 2008) and in basophils during the first hours of ultra-rush venom immunotherapy (Novak et al. 2012). H2R upregulation is responsible for the inhibition of IL-4 and the stimulation of IL-10 secretion by IL-4^+ T cells (Meiler et al. 2008) as well as the inhibition of histamine release and cytokine secretion from basophils (Novak et al. 2012; Lichtenstein and Gillespie 1973). In addition, activation of H2R inhibits histamine release from rodent mast cells (Masini et al. 1982), neutrophil activation (Burde et al. 1989), eosinophil chemotaxis (Clark et al. 1975) and degranulation (Ezeamuzie and Philips 2000), γδ T cell-mediated cytotoxicity (Truta-Feles et al. 2010), and reduces the inflammatory response of dendritic cells to microbial ligands (Frei et al. 2013; Mazzoni et al. 2003). Interestingly, histamine via H2R protects natural killer cells from myeloid cells-dependent inactivation and fosters their killing of human acute myeloid leukemia blasts (Brune et al. 1996).

H3R is expressed mainly in the central nervous system (Sadek et al. 2016). Accordingly, H3R knockout mice exhibit a neurological phenotype: decrease in

locomotor activity, wheel-running behavior, and body temperature (Toyota et al. 2002). In addition, mild obesity (Takahashi et al. 2002) and reduction in anxiety (Rizk et al. 2004) were reported in these mice. Levi and collaborators reported the presence of H3R on sympathetic nerve terminals in the human heart (Imamura et al. 1995). Activation of this receptor leads to the attenuation of norepinephrine release in conditions associated with enhanced adrenergic activity, such as acute myocardial ischemia (Imamura et al. 1994). Moreover, activation of H3R inhibits norepinephrine release during protracted myocardial ischemia (Imamura et al. 1996).

H4R has limited homology with the other histamine receptors and is preferentially expressed on immune cells, namely T cells (Truta-Feles et al. 2010; Gantner et al. 2002; Gutzmer et al. 2009; Leite-de-Moraes et al. 2009; Morgan et al. 2007), NK cells, dendritic cells (Damaj et al. 2007), eosinophils (Buckland et al. 2003; O'Reilly et al. 2002), basophils (Shiraishi et al. 2013), and mast cells (Thurmond et al. 2004; Godot et al. 2007; Hofstra et al. 2003). Interestingly, this receptor modulates immune cell chemotaxis as well as several other functions of these cells. At variance with mast cells from wild type mice, mast cells from H4R knockout mice do not migrate in response to histamine (Hofstra et al. 2003). H4R antagonism prevents histamine-induced $[Ca^{2+}]_i$ increase, mast cell chemotaxis, and submucosal mast cell accumulation in the trachea of mice after histamine inhalation (Thurmond et al. 2004). Histamine acting through H4R enhances C-X-C motif chemokine (CXCL) 12-induced chemotaxis of mast cell precursors, but not mature mast cells (Godot et al. 2007). H4R can impair cardiac mast cell renin release in a model of ischemia/reperfusion (Aldi et al. 2014). A role for H4R was also demonstrated in the modulation of eosinophil and basophil chemotaxis in response to histamine (Buckland et al. 2003; O'Reilly et al. 2002; Shiraishi et al. 2013). In addition, H4R activation reduces basophil expression of CD63 and CD203c and the production of sulfidoleukotrienes following FcεRI cross-linking (Mommert et al. 2016). Interestingly, the involvement of H4R in the development of allergic disorders has also been shown in vivo. For example, in a mouse model of allergic rhinitis histamine released from mast cells recruits H4R-expressing basophils to the nasal cavity, an event that is required for the development of early or late phase nasal responses following allergen challenge (Shiraishi et al. 2013). Combined treatment with H1R and H4R antagonists in the challenge phase prevents the development of diarrhea and intestinal inflammation in an experimental model of peanut sensitization and challenge, probably by affecting dendritic cell chemotaxis and function (Wang et al. 2016). H4R knockout mice develop less skin lesions compared with wild type mice in an experimental model of atopic dermatitis, although pharmacological blockade of H4R is required during both sensitization and challenge to partially mimic the results observed in H4R knockout mice (Rossbach et al. 2016). H4R might also contribute to skin allergic inflammation by activating Th2 cells and inducing pruritus via IL-31 (Gutzmer et al. 2009). Nevertheless, in a murine model of allergic asthma intratracheal administration of the H4R agonist 4-Methylhistamine mitigated airway inflammation, probably by inducing the recruitment of $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cells (Morgan et al. 2007).

These results highlight the complex role of H4R in allergic inflammation. Interestingly, H4R has also been involved in the pathogenesis of non-allergic disorders by affecting multiple cell types. H4R blockade decreases neutrophil accumulation in experimental models of peritonitis (Thurmond et al. 2004) and pleurisy (Takeshita et al. 2003). H4R activation induces chemotaxis of IL-2-activated NK cells, dendritic cells, THP-1 cells (a human acute monocytic leukemia cell line) (Damaj et al. 2007), γδ T cells (Truta-Feles et al. 2010), and enhances cytokine secretion from invariant NK T (iNKT) (Leite-de-Moraes et al. 2009).

4 Mast Cell and Basophil Degranulation and Histamine Release

Mast cell and basophil degranulation and histamine release is a complex process that can be initiated and modulated by IgE-dependent and non-IgE-dependent stimuli activating a wide variety of receptors (Fig. 2), including cytokines like IL-3, IL-33 and SCF and TLR agonists that can also enhance the response to other stimuli (Stone et al. 2010; Marone et al. 2005; Galli and Tsai 2012; Borriello et al. 2014b; Voehringer 2012; Schroeder 2011). Cross-linking of FcεRI-bound IgE induced by antigens, superantigens, and the histamine-releasing factor (which bind a relatively large fraction of IgE and IgG on the Fab portions) (Kawakami et al. 2014) results in the release of histamine as well as other factors, including lipid mediators, cytokines, and chemokines. A key singling protein involved in this process is the cytosolic spleen tyrosine kinase (Syk) that induces the phosphorylation of adaptor and signaling molecules (Borriello et al. 2014b; Havard et al. 2011; Kepley et al. 1999; Lavens-Phillips and MacGlashan 2000; MacGlashan 2007). An important target of Syk is the Tec family Bruton's tyrosine kinase (Btk). Indeed, Btk inhibition blocks mast cell degranulation and IgE-mediated basophil activation (MacGlashan et al. 2011; Hata et al. 1998; Kuehn et al. 2008). Mast cell and basophil activation can also be inhibited by negative regulators of signaling pathways. For example, the lipid phosphatase SHIP-1 dephosphorylates the inositol ring of phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) to yield phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), thereby reducing calcium influx and cell activation (Huber and Gibbs 2015).

Mast cell and basophil histamine release is also modulated by other factors, like substance P, complement anaphylatoxins C3a and C5a, endothelin 1, formyl-methionyl-leucyl phenylalanine (fMLF), many of them acting through GPCRs (Maurer et al. 2004; Schafer et al. 2013; Yano et al. 1989; Grant et al. 1975; Siraganian and Hook 1977). Interestingly, mast cells express the MAS-related G protein-coupled receptor (MRGPR) B2 (mouse) or X2 (human) that binds to and mediates mast cell activation in response to anti-microbial peptides, basic secretagogues (e.g., substance P, mastoparan, compound 48/80) as well as the peptidergic drug icatibant, neuromuscular blocking drugs, and fluoroquinolones (McNeil et al. 2015; Subramanian et al. 2011, 2013; Kashem et al. 2011). Mast cell degranulation events in response to FcεRI crosslinking and MRGPRB2 or X2 (as well as other

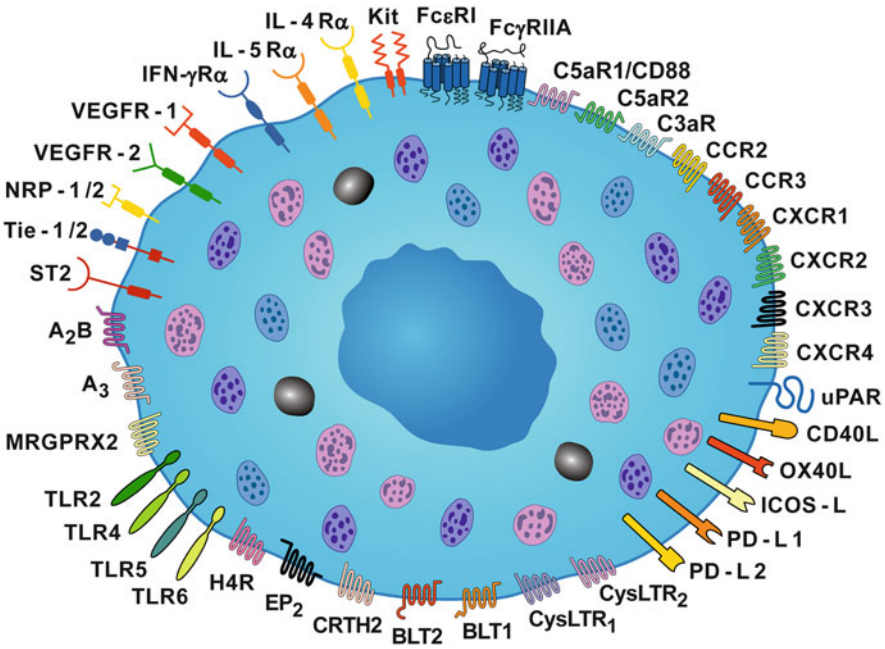

Fig. 2 Surface receptors expressed by human mast cells. Human mast cells express the tetrameric high affinity receptor for IgE (FcεRI) and the FcγRIIA, and their cross-linking induces the release of proinflammatory and immunomodulatory mediators. Mast cells express the KIT receptor (CD117), which is activated by stem cell factor (SCF). These cells express a plethora of receptors such as Toll-like receptor (TLR) 2, TLR4, TLR5, TLR6, receptors for chemokines (CCR2, CCR3, CXCR1, CXCR2, CXCR3, and CXCR4), two receptors for cysteinyl leukotrienes (CysLTR1 and CysLTR2), two leukotriene B_4 receptors (BLT1 and BLT2), the prostaglandin D_2 receptor (CRTH2), the prostaglandin E_2 receptor (EP_2), two adenosine receptors (A_{2B} and A_3), and histamine H4 receptor (H4R). Mast cells express receptor for various cytokines (IL-4Rα, IL-5Rα, IFN-γRα, ST2) and the MAS-related G protein coupled receptor (MRGPRX2). These cells also express receptors for vascular endothelial growth factors (VEGFR1 and VEGFR2), and VEGFR co-receptors, neuropilin-1 and neuropilin-2 (NRP1 and NRP2), for anaphylatoxins (C5aR1/CD88, C5aR2 and C3aR), and the high affinity urokinase plasminogen activator receptor (uPAR). Human mast cells also express co-receptors for T-cell activation [CD40 ligand (CD40L), tumor necrosis factor superfamily member 4 (OX40L), inducible costimulator ligand (ICOS-L), programmed death ligands (PD-L1 and PD-L2)]

GPCRs) activation are qualitatively and quantitatively different. FcεRI crosslinking induces a slower but sustained Ca^{2+} response compared to MRGPRX2 activation and is associated with granule fusion and the release of PGE_2, cytokines, and vascular endothelial growth factors (VEGFs). On the other hand, MRGPRX2-induced activation is rapid and associated with a transient Ca^{2+} response. Inhibition of IκB kinase-β (IKK-β) converted the FcεRI-induced degranulation phenotype to the MRGPRX2-mediated degranulation phenotype. Of note, the different mast cell degranulation profiles were also confirmed in vivo following FcεRI and MRGPRB2

activation (Gaudenzio et al. 2016). Two different modes of degranulation and histamine release that likely require distinct pathways and calcium signaling have also been described for basophils: the anaphylactic degranulation that consists in rapid morphologic changes and exocytosis of intracellular granules and is associated with up-regulation of CD63 (Knol et al. 1991; MacGlashan 2010); the piecemeal degranulation that consists in granule content secretion without exocytosis and may be associated with CD203c up-regulation (MacGlashan 2012; Buhring et al. 2004).

Mast cell and basophil degranulation and histamine release may also be hindered by the concurrent activation of inhibitory receptors (Fig. 3). Inhibitory receptors can be divided into the Ig receptor and the C-type (calcium dependent) lectin superfamilies and are characterized by immunoreceptor tyrosine-based inhibition motifs (ITIMs) that downregulate the activation signals transmitted through immunoreceptor tyrosine-based activation motifs (ITAMs). Upon activation of ITIM-containing receptors, tyrosine residues within the motifs become phosphorylated. This leads to the recruitment of phosphatases, namely tyrosine phosphatases SHP-1 and SHP-2 and lipid phosphatase SHIP-1. SHP-1/2 inhibits the action of tyrosine kinase, while SHIP-1 terminates the phosphoinositide 3-kinase (PI3K)-mediated

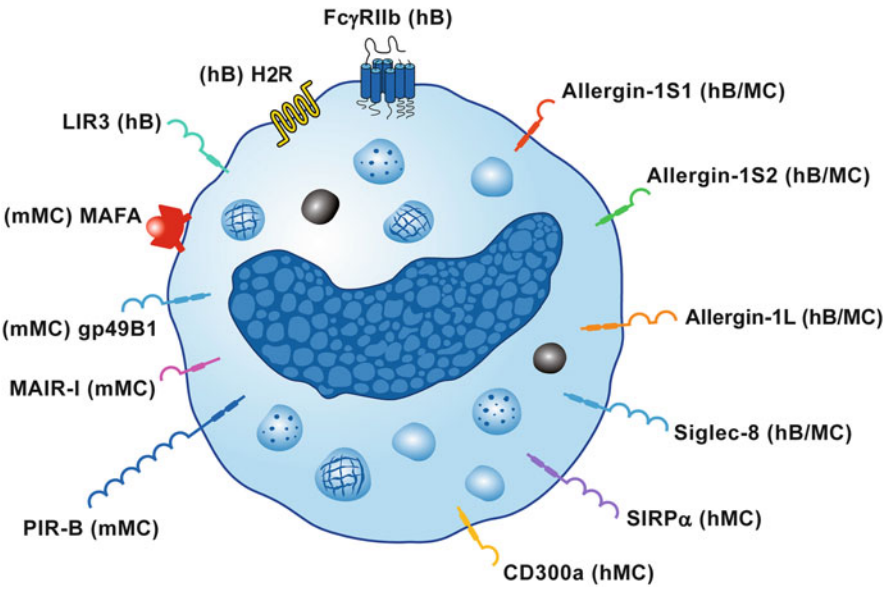

Fig. 3 Inhibitory receptors expressed by human mast cells (hMC) and basophils (hB) and murine mast cells (mMC). Human basophils and mast cells share the expression of three inhibitory allergin receptors (Allergin-1S1, Allergin-1S2, and Allergin-1L) and sialic acid immunoglobulin (Ig)-like lectins (Siglec)-8. Human mast cells express signal-regulatory protein-α [SIRPα] and CD300a, whereas human basophils express FcγRIIb, histamine H2 receptor (H2R), and leukocyte Ig-like receptor (LIR3). Mouse mast cells express paired Ig-like receptor B (PIR-B), myeloid-associated Ig-like receptor (MAIR)-I, the mast cell function associated antigen (MAFA) and gp49B1

pathway (Daeron et al. 2008; Karra and Levi-Schaffer 2011). Several inhibitory receptors on mast cells and basophils have been characterized, including FcγRIIB, CD300, and sialic acid binding Ig-like lectin (Siglec)-8.

FcγRIIB is a low affinity IgG receptor that can inhibit IgE-mediated responses of both mast cells and basophils (Macglashan et al. 2014; Zhu et al. 2002). Co-aggregation of FcγRIIB and FcεRI suppresses FcεRI-mediated activation. Interestingly, a chimeric protein composed of key portions of the human γ1 and the human ε immunoglobulin heavy chains can inhibit FcεRI-mediated activation of human basophils in vitro and FcεRI-mediated degranulation of murine mast cell expressing the human FcεRI in an in vivo model of passive cutaneous anaphylaxis.

The CD300 molecules are a family of immunoglobulin receptors that includes activating (CD300b, CD300e) and inhibitory (CD300a, CD300f) members (Clark et al. 2009). Mast cells express CD300a and CD300f and their respective murine orthologs LMIR1 and LMIR3 (Kumagai et al. 2003). LMIR1/CD300a cross-linking inhibits both FcεRI-dependent and SCF-dependent signaling (Bachelet et al. 2005). Interestingly, bispecific antibodies that co-aggregate LMIR1/CD300a with either FcεRI or KIT (CD117) inhibit allergic responses in vivo (Bachelet et al. 2006, 2008). LMIR3/CD300f binding to its ligands ceramide and sphingomyelin inhibits FcεRI-mediated activation of mast cells in vitro and in vivo (Izawa et al. 2012, 2014). Basophils also express CD300a in the peripheral blood of both healthy and allergic subjects, and its activation inhibits IgE-mediated anaphylactic degranulation (Sabato et al. 2012, 2014; Gibbs et al. 2012).

Siglecs are a group of sialic acid-binding cell surface proteins predominantly expressed by immune cells. In particular, Siglec-8 is expressed on human eosinophils, mast cells, and to a lesser extent on basophils (Kiwamoto et al. 2012). Siglec-8 cross-linking inhibits FcεRI-dependent histamine and PGD_2 release from human mast cells (Yokoi et al. 2008). However, mouse mast cells do not express Siglec-F, which makes it difficult to understand its function on these cells in vivo.

5 Biological Effects of Mast Cell and Basophil Histamine Release

Mast cells and basophils have been involved in several pathophysiological conditions. Since these cells release a variety of preformed and de novo synthesized mediators, a specific role for mast cell- or basophil-derived histamine has not always been identified. Mast cell-derived histamine plays an important role in conditions associated with vascular leakage like urticaria and anaphylaxis (Meyer et al. 2013; Cohen and Rosenstreich 1986; Lieberman and Garvey 2016). Its involvement in other disorders like atopic dermatitis, asthma, and rheumatoid arthritis might be supported by the pre-clinical results showing that genetic or pharmacological blockade of H4R ameliorates these conditions (Liu 2014). Whether H4R activation in these models relies on mast cell- or basophil-derived histamine has still to be demonstrated. Indeed, in a model Th2-dependent skin

inflammation H4R blockade was effective in reducing itch and edema even in mast cell-deficient mice (Cowden et al. 2010).

Mast cell- and basophil-derived histamine may mediate the communication with other cell types. For example, in a mouse model of allergic rhinitis histamine released following IgE-mediated activation of mast cells recruits H4R-expressing basophils to the nasal cavity, an event that was required for the development of early or late phase nasal responses following allergen challenge (Shiraishi et al. 2013). Mast cell-derived histamine enhances the proliferation and activation of cholangiocytes and hepatic stellate cells, an event that might be relevant for the development of sclerosing cholangitis (Jones et al. 2016). Moreover, human dermal mast cell-derived tumor necrosis factor (TNF)-α and histamine increase CXCL8/IL-8 expression in human melanoma cell lines (Artuc et al. 2011). Mast cells activated by IL-33 and immune complexes release IL-10 and histamine that in turn inhibit LPS-mediated monocyte activation (Rivellese et al. 2015). Similarly, monocyte activation can also be restrained by basophil-derived histamine released upon IL-33 stimulation and FcεRI-crosslinking (Rivellese et al. 2014), while monocyte alternative activation relies on basophil-derived IL-4 and IL-13 following IL-3 stimulation and FcεRI-crosslinking (Borriello et al. 2015). Basophil-derived histamine also enhances IL-17 production by memory CD4 T cells at least in part by activating H2R and H4R on T cells (Wakahara et al. 2012). Interestingly, basophil histamine release is altered in some clinical conditions. For example, basophils isolated from patients with food allergy or severe asthma show spontaneous histamine release in vitro (May 1976; Sampson et al. 1989; Schroeder et al. 2013; Findlay and Lichtenstein 1980). IgE-mediated basophil histamine release is reduced in chronic idiopathic urticaria (CIU) patients (Kern and Lichtenstein 1976). In particular, CIU patients can be classified as responders (CIU-R) or non-responders (CIU-NR) on the basis of basophil histamine release in response to anti-IgE ($>10\%$ or $<10\%$ of cellular histamine content, respectively). There is evidence that the pattern of basophil IgE-mediated histamine release observed in these patients results from altered FcεRI-mediated signaling (Saini 2009; Vonakis and Saini 2008; Vonakis et al. 2007).

6 Conclusions

Several stimuli can induce or modulate mast cell and basophil histamine release. Although the pathophysiological relevance of this phenomenon has been demonstrated in some pre-clinical or clinical disorders, the discovery of H4R expressed mainly on immune cells has uncovered new roles for histamine (possibly derived from mast cells and basophils) in a wider range of inflammatory and autoimmune disorders. Moreover, the identification and characterization of inhibitory receptors expressed by mast cells and basophils as well as distinct modalities of mediator release upon triggering of different classes of receptors may uncover new therapeutic approaches for modulating mast cell and basophil degranulation and histamine release.

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Histamine H_2 Receptor in Blood Cells: A Suitable Target for the Treatment of Acute Myeloid Leukemia

Federico Monczor, Sabrina Copsel, Natalia Fernandez, Carlos Davio, and Carina Shayo

Contents

Abstract

Acute myeloid leukemia (AML) consists in a cancer of early hematopoietic cells arising in the bone marrow, most often of those cells that would turn into white blood cells (except lymphocytes). Chemotherapy is the treatment of choice for AML but one of the major complications is that current drugs are highly toxic and poorly tolerated. In general, treatment for AML consists of induction chemotherapy and post-remission therapy. If no further post-remission is given, almost all patients will eventually relapse. Histamine, acting at histamine type-2 (H_2) receptors on phagocytes and AML blast cells, helps prevent the

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production and release of oxygen-free radicals, thereby protecting NK and cytotoxic T cells. This protection allows immune-stimulating agents, such as interleukin-2 (IL-2), to activate cytotoxic cells more effectively, enhancing the killing of tumor cells. Based on this mechanism, post-remission therapy with histamine and IL-2 was found to significantly prevent relapse of AML. Alternatively, another potentially less toxic approach to treat AML employs drugs to induce differentiation of malignant cells. It is based on the assumption that many neoplastic cell types exhibit reversible defects in differentiation, which upon appropriate treatment results in tumor reprogramming and the induction of terminal differentiation. There are promissory results showing that an elevated and sustained signaling through H_2 receptors is able to differentiate leukemia-derived cell lines, opening the door for the use of H_2 agonists for specific differentiation therapies. In both situations, histamine acting through H_2 receptors constitutes an eligible treatment to induce leukemic cell differentiation, improving combined therapies.

Keywords
Histamine • Acute myeloid leukemia • Chemotherapy • Cell differentiation

1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells arising in the bone marrow that fail to differentiate, to respond to normal regulators of proliferation, and that do not undergo programmed cell death or apoptosis. Leukemic cells that interfere with normal hematopoiesis can escape into the peripheral blood and result in organ infiltration, most threateningly the CNS and lung. This malignant alteration is characterized by a loss of normal hematopoietic function leading to bone marrow failure that is the most common underlying cause of death. The genetic reprogramming of leukemic cells renders them ineffective at generating mature neutrophils, monocytes, red cells, and platelets. Thus, the main sign of bone marrow failure is infection caused by a large range of pathogens including gram-positive and gram-negative bacteria, *Candida* species, and *Aspergillus* species (Anderlini et al. 1996).

It has been estimated about 20,830 new cases of AML and 10,460 deaths only in the United States for 2015 (Siegel et al. 2015). AML is more common in the elderly, with a median age at diagnosis of 67 years, but it represents 15–20% of childhood acute leukemias (Pui et al. 2004). Risks factors for acquiring AML include exposure to ionizing radiation, benzene, and cytotoxic chemotherapy. Almost 15% of patients with AML develop the disorder after the use of chemotherapy for solid cancer treatment.

There are two main systems that have been used to classify AML: The French-American-British (FAB) classification and the World Health Organization (WHO) classification. Depending on the cell type from which leukemia develops and how

Table 1 WHO classification of AML

AML with certain genetic abnormalities	AML with a t(8;21) RUNX1-RUNX1R1
	AML with a t(16;16) or inv(16) CBFB-MYH11
	AML with a t(9;11) MLL-AF9
	APL (M3) with a t(15;17) PML-RARA
	AML with a t(6;9) DEK-NUP214
	AML with a t(3;3) or inv(3) EVI1-RPN1
	AML (megakaryoblastic) with a t(1;22) RBM15-MKL1
AML with myelodysplasia-related changes *AML related to previous chemotherapy or radiation* *AML not otherwise specified* Cases of AML that do not fall into one of The above groups and is similar to the FAB classification	Undifferentiated AML (M0)
	AML without maturation (M1)
	AML with maturation (M2)
	Acute myelomonocytic leukemia (M4)
	Acute monocytic leukemia (M5)
	Acute erythroid leukemia (M6)
	Acute megakaryoblastic leukemia (M7)
	Acute basophilic leukemia
	Acute panmyelosis with fibrosis
Myeloid sarcoma	Also known as granulocytic sarcoma or chloroma
Myeloid proliferations related to down syndrome *Undifferentiated and biphenotypic acute leukemias*	Leukemias that have both lymphocytic and myeloid features
	Also called ALL with myeloid markers, AML with
	Lymphoid markers, or mixed phenotype acute leukemias

t translocation, *inv* inversion

mature the cells are, FAB system divides AML into subtypes from M0 to M7. On the other hand, WHO classification is newer and defines subsets of AML based on morphologic and cytogenetic characteristics (Table 1).

AML treatment options depend on the subtype as well as on the prognostic features. However, in the last decades, chemotherapy has been the treatment of choice, sometimes followed by allogeneic hematopoietic stem cell transplantation. One of the major complications of chemotherapy is that the current drugs are highly toxic and poorly tolerated, especially by older patients (Estey and Döhner 2006). In general, treatment for AML consists of induction chemotherapy (combination of cytarabine and the anthracycline drugs), less frequently central nervous system prophylaxis (to prevent CNS relapse), and post-remission therapy. Up to 70% of patients will achieve remission with the induction protocol; however, if no further post-remission is given, almost all patients will eventually relapse. Remission rates and overall survival depend on different features among them: age of the patient, cytogenetics (chromosomal aberration), secondary molecular changes within the leukemic clone, previous bone marrow disorders (e.g., myelodysplasia), and comorbid illnesses.

Over the last few decades, the concept of differentiation therapy, whereby immature cells may be stimulated to develop into their mature phenotype, aroused considerable interest. Many efforts are in progress to evaluate new differentiation drugs for the treatment of leukemia in which early hematopoietic progenitors appear to exhibit maturation arrest. Treatment of acute promyelocytic leukemia (APL) with the differentiation agents, vitamin A metabolite all-trans-retinoic acid (ATRA) (Nowak et al. 2009) or arsenic trioxide (As_2O_3) (Chou et al. 2005), has been successfully applied. In addition, factors that increase cAMP-mediated signaling, such as cyclic nucleotide phosphodiesterase (PDE) inhibitors, augment the ability of these approved therapies to induce differentiation in APL blast cells (Lerner and Epstein 2006).

2 H_2 Receptor Signaling and Physiology

The fact that classic antihistamines were not able to block histamine-induced gastric secretion led the researchers to hypothesize the existence of a new histamine receptor subtype (Ash and Schild 1966). Some years later, this hypothesis was confirmed after the development of specific ligands able to block gastric acid secretion (Black et al. 1972), naming this new receptor subtype as H_2 receptor.

Numerous studies had found that H_2 receptors act as potent stimulators of intracellular cAMP accumulation (Leurs et al. 1995; Hill et al. 1997; Panula et al. 2015). It has been demonstrated that the modulation of cAMP levels occurs via the coupling and activation of Gαs G-protein subunit. This was experimentally demonstrated by [α-^{32}P]GTP labeling of Gαs subunits after receptor stimulation in mammalian and insect cell expression systems (Kühn et al. 1996; Leopoldt et al. 1997), by using receptor-G-protein fusion chimeras, [^{35}S]GTPγS binding, and steady-state GTP hydrolysis (Kelley et al. 2001; Wenzel-Seifert et al. 2001).

In addition to Gαs coupling to adenylyl cyclase, H_2 receptors couple to other signaling systems. It has been shown that H_2 receptors couple also to Gαq/11 proteins, resulting in inositol phosphate formation and increases in cytosolic Ca^{2+} concentration in some H_2 receptor-expressing cells. Experiments equivalent to those used to demonstrate receptor coupling to Gαs have shown that H_2 receptor can also activate Gαq proteins in both mammalian and insect cells (Kühn et al. 1996; Leopoldt et al. 1997). In gastric parietal cells, HL-60 cells, and hepatoma-derived cells transfected with the canine H_2 receptor cDNA, H_2 receptor stimulation has been shown to increase the intracellular free concentration of calcium ions (Chew 1985, 1986; Malinowska et al. 1988; Mitsuhashi et al. 1989; Chew and Petropoulos 1991; Delvalle et al. 1992; Seifert et al. 1992). Interestingly, H_2 receptor coupling to Gαq has been found in rat mammary carcinoma and undifferentiated rat mammary cells and in human breast epithelial cell lines. In these cases, the alternate coupling was correlated with the differentiation cell stage suggesting a relationship between H_2 receptor coupling to Gαq and the loss of a regulatory mechanism of cell growth (Davio et al. 1995a, b, 2002).

In addition, in CHO cells transfected with the rat but not human H_2 receptor, receptor stimulation produces both an increase in cAMP accumulation and an inhibition of P2u-receptor-mediated arachidonic acid release (Traiffort et al. 1992; Leurs et al. 1994). These observations suggest that these effects might depend on the level of receptor expression or subtle differences between clonal cell lines.

As many other GPCRs, H_2 receptor signaling is tightly regulated by receptor desensitization and internalization after agonist stimulation (Smit et al. 1996; Fukushima et al. 1997). Desensitization of the H_2 receptor involves both GPCR kinases GRK-2 and GRK-3 but not GRK-5 or GRK-6 (Rodriguez-Pena et al. 2000; Shayo et al. 2001). Remarkably, the regulation of the H_2 receptor by GRK-2 relies on a dual mechanism, while the kinase activity is implicated in receptor internalization and recycling, the RGS (regulator of G-protein signaling) homology domain of GRK-2 is responsible for H_2 receptor desensitization (Fernandez et al. 2011).

Interestingly, GRK-2-mediated desensitization has proved to be involved in the lack of hematopoietic cell maturation promoted by H_2 receptor stimulation. When GRK-2 is downregulated, H_2 receptor-mediated cAMP response is higher and more sustained, allowing cells to differentiate after treatment with H_2 agonists (Fernández et al. 2002). This fact results therapeutically relevant and will be extensively discussed later. Concerning receptor internalization, a role of dynamin, β-arrestin, and clathrin has also been reported (Fernandez et al. 2008), and the GTPase dynamin has been identified as a binding partner for the H_2 receptor, both in vitro and in vivo (Xu et al. 2008).

Regulation of gastric acid secretion represents the paradigmatic function of histamine that is mediated by the activation of H_2 receptors. However, along the years, several other functions of histamine were assigned to its action over H_2 receptor. In addition to the stomach, the H_2 receptor is expressed in the brain, smooth and cardiac muscle cells, chondrocytes, endothelial and epithelial cells, neutrophils, eosinophils, monocytes, macrophages, dendritic cells, and T and B cells (Jutel et al. 2009).

Histamine has been typically considered an effector molecule for chronic and immediate hypersensitivity (Pearce 1991). However, growing evidence suggest that it is a potent modulator of the immune system. At low physiological concentrations, histamine can act as an immunostimulant exerting its action mainly through H_1 receptors. On the other hand, at higher concentrations, histamine released by basophils, mastocytes, or tumor cells acts as immunosuppressor through H_2 receptors, activating suppressor T cells and inhibiting T helper cytokine production (Jutel et al. 2006). Histamine also inhibits the production of reactive oxygen species (ROS) in isolated monocytes, neutrophils, and leukemic cells recovered from patients with myelomonocytic and monocytic forms of AML (FAB classes M4 and M5) (Hellstrand et al. 1994; Ching et al. 1995; Reher et al. 2012; Aurelius et al. 2012; Werner et al. 2014). This effect on ROS production has a great impact on clinical use of H_2 ligands to treat hematopoietic-related malignancies in general and AML in particular and will be further discussed. Remarkably, it has been reported that the effect on the oxidative burst of granulocytes and monocytes is

not mediated by cAMP accumulation, and it has been provided substantial evidence for ligand-specific conformations of the H_2 receptor, suggesting that H_2 receptor-biased signaling might be an important concept to consider for clinical treatment design (Reher et al. 2012; Werner et al. 2014).

3 Histamine Dihydrochloride and H_2 Agonists for the Treatment of Acute Myeloid Leukemia

Signs and symptoms of AML are caused by the lack of normal blood cells and their replacement with leukemic cells. Although the leukemic cells themselves are derived from white blood cell precursors, they have no infection-fighting capacity, and therefore AML makes the patient susceptible to infections (Anderlini et al. 1996).

The pathophysiology of AML permits to envisage at least two treatment strategies, the most obvious and conventional involves chemotherapy, aiming to kill malignant cells. However, due to the high toxicity and lack of specificity of most chemotherapeutic agents, an alternative therapy has been suggested based on the possibility of differentiate abnormal undifferentiated malignant cells to their differentiated counterparts. This strategy allows acquiring the lineage specificity and functional characteristics of mature cells. This approach is termed "differentiation therapy" and is based on the hypothesis that many neoplastic cell types exhibit reversible defects in the course of differentiation, which, upon appropriate treatment, result in tumor reprogramming with a concomitant loss of proliferative capacity and induction of terminal differentiation or apoptosis (Nowak et al. 2009).

As discussed before, histamine receptors have a role in immune cell life cycle and differentiation (Jutel et al. 2006, 2009), making them suitable targets for the treatment of AML. With varied results, both strategies are in different steps of development. They are depicted in Fig. 1 and will be discussed below.

3.1 Histamine Dihydrochloride as Chemotherapy Complement

AML first-line treatment is primarily chemotherapy that is divided into two phases: induction and post-remission (or consolidation) therapy. The goal of the first phase is to reach a complete remission, meaning that no disease can be detected with available diagnostic methods (i.e., to reduce the number of leukemic cells to an undetectable level). The length of remission depends on the prognostic features of the original leukemia, and although chemotherapy induces remission in up to 80% of patients with de novo AML, in general, all remissions will fail without additional consolidation therapy (Grimwade et al. 1998, 2001; Farag et al. 2006). Therefore, more therapy is necessary to eliminate non-detectable malignant cells and prevent relapse, that is, to achieve a cure.

Natural killer (NK) cells are an important component of the innate immune system, providing first-line defense against virus-infected cells and tumors. NK

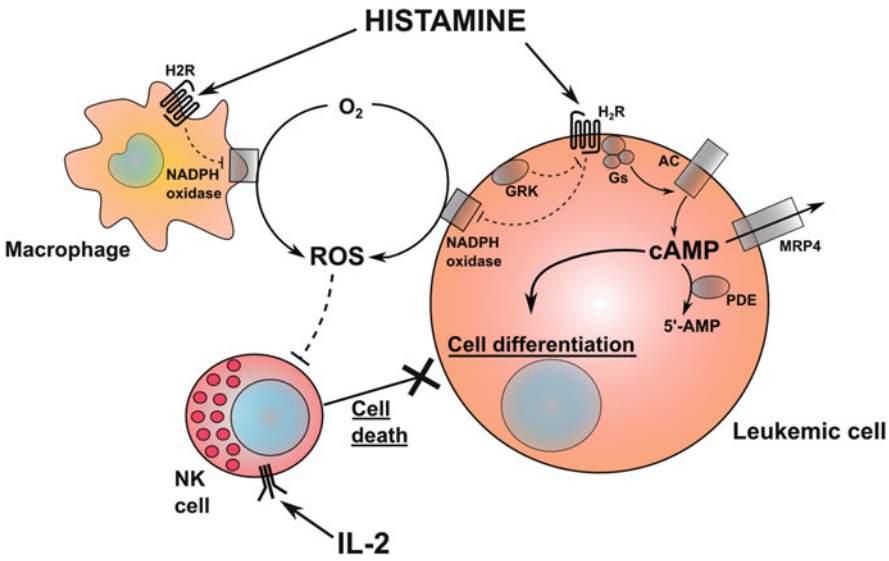

Fig. 1 Histamine actions on leukemic cell fate. Histamine or H_2 agonists increasing intracellular cAMP levels are able to induce leukemic cell differentiation. To achieve the effect, GRK-2-mediated H_2 receptor desensitization, PDE-mediated cAMP degradation, and/or MRP4-mediated second messenger efflux should be inhibited. Histamine is also able to inhibit macrophage and leukemic cells ROS production allowing IL-2 activation of NK cells or T cytotoxic cells with the consequent leukemic cell death. *Arrows* indicate activation, while *dotted lines* indicate inhibition

cells are cytotoxic to AML blasts as demonstrated by the graft-versus-leukemia effect in patients with leukemia after bone marrow transplantation (Lotzová et al. 1987; Barrett 2008), and higher NK-mediated cytotoxicity has been reported to result in higher leukemia-free survival (Lowdell et al. 2002). Moreover, there are studies suggesting that NK cells may be compromised in AML (Costello et al. 2002; Fauriat et al. 2007). These observations support the role of these cells in AML progression and are suggestive about their prognostic value helping to the accurate prediction of disease outcome.

In this regard, interleukin-2 (IL-2) is a key cytokine in the activation of T and NK cells (Waldmann 2006), and it is indicated for the treatment of metastatic renal cell carcinoma and metastatic melanoma (Proleukin®, Novartis Pharmaceuticals Corporation). However, any significant advantage of the use of IL-2 over no treatment could not be demonstrated in large, randomized trials in patients with AML (Blaise et al. 2000; Baer et al. 2008; Pautas et al. 2010; Kolitz et al. 2014). This lack of in vivo efficacy in patients can be attributed to "tumor-induced immunosuppression" of NK cells (Hellstrand 2002). Tumor-associated macrophages and leukemic cells recovered from patients with myelomonocytic and monocytic forms of AML (FAB classes M4 and M5) convert oxygen into ROS, and these free radicals create a reduced environment that impedes the activation of

NK cells, including that by IL-2 (Murdoch et al. 2004; Romero et al. 2009; Aurelius et al. 2012).

The mechanism of action of histamine through H_2 receptors in AML consists in the inhibition of the activity of NADPH oxidase and the consequent production of ROS by tumor-associated monocytes and by leukemic cells themselves, conferring protection from tumor-induced immunosuppression (Hellstrand 2002; Romero et al. 2009; Martner et al. 2010). Therefore, the addition of histamine dihydrochloride to IL-2 enables the activation of T cells and NK cells by IL-2 (Hellstrand et al. 1994). In vitro studies have shown that this effect of histamine is mimicked by the H_2-specific agonist dimaprit and blocked by the addition of the H_2 antagonists ranitidine or cimetidine (Hellstrand and Hermodsson 1986; Brune et al. 1996). It is worth noting that IL-2 plays also a crucial role in Treg cells boosting immune regulation. IL-2-dependent activation of NK and T cytotoxic cells depends on the application of high doses of IL-2, while infusion of relatively low doses of IL-2 seems to selectively produce Treg cells boosting immune suppressive mechanisms (Malek and Bayer 2004). This balance between immune activation and suppression by IL-2 should be considered when therapeutic schemes are to be developed. In addition, it has been described that histamine acting on H_2 receptors, and independently of cAMP modulation, is able to decrease the high constitutive activity of Akt2 in U937 cells (Werner et al. 2016). These observations are very significant considering that phosphorylation of this kinase is crucial for the regulation of numerous downstream targets involved in cell growth, proliferation, survival, differentiation, and metabolism (Martelli et al. 2009; Vivanco and Sawyers 2002). Since Akt activation promotes AML progression (Martelli et al. 2006; Vivanco and Sawyers 2002) and it is associated with a shorter overall survival (Gallay et al. 2009; Min et al. 2003), it cannot be discarded the inhibition of Akt2 activation as a complementary mechanism by which histamine achieves its effects as a potential clinical treatment for post-remission therapy.

As a widely distributed local mediator and neurotransmitter, histamine acts on a multitude of cell types in addition to cells of the immune system and blood cells, including smooth muscle cells, neurons, and endocrine and exocrine cells, having many systemic effects, mediated mainly by H_1 and H_2 receptors such as anaphylaxis, vasodilation, gastric acid secretion, and neurotransmission (Panula et al. 2015). Consequently, the use of subcutaneous histamine dihydrochloride may result in vasodilation and hypotension and other related adverse events.

Information about the tolerability of histamine dihydrochloride with or without concomitant IL-2 was obtained from the phase III trial in patients with AML in complete remission (Brune et al. 2006), reviews (Mekhail et al. 2000), and the EU summary of product characteristics (http://www.ema.europa.eu). Since histamine is a potent vasoactive agent, the use of histamine dihydrochloride has been frequently (>30%) associated with flushing, headache, fatigue, and pyrexia (Hellstrand 2002). Other less frequent vasodilatation-related adverse events include hypotension and tachycardia (Martner et al. 2010). Because histamine dihydrochloride and IL-2 are administered by subcutaneous injection, injection-site adverse events such as injection-site granuloma and erythema may occur, and this type of reaction is the

most common cause of dose reduction or treatment interruption (Brune et al. 2006). Anyway, in the phase III trial, combined histamine dihydrochloride and IL-2 therapy had an acceptable tolerability profile.

At his point, histamine tolerability needs to be established in a wider AML population, not restricted to stringent clinical trial inclusion criteria, and over the longer term. Moreover, the use of specific H_2 agonists instead of histamine could constitute a genuine strategy to avoid undesired effects produced by the activation of other histamine receptor subtypes. In conclusion, histamine dihydrochloride and IL-2 as post-consolidation immunotherapy significantly prolonged leukemia-free survival compared with no treatment having an acceptable tolerability profile and seems to be a useful therapy option for adult patients with AML in remission.

3.2 H_2 Histamine Ligands as Leukemic Differentiation Agents

As mentioned before, the aim of differentiation therapy is to reprogram neoplastic cells with a treatment that suppresses the exacerbated proliferative capacity of tumor cells and induces terminal differentiation or apoptosis. Differentiation induction as a therapeutic strategy has the greatest impact on hematopoietic malignancies, most notably on leukemia.

Treatment of acute promyelocytic leukemia with differentiation agents such as vitamin A metabolite all-trans-retinoic acid (ATRA) (Nowak et al. 2009) or arsenic trioxide (As2O3) (Chou et al. 2005) has been successfully applied. In addition, factors that increase cAMP-mediated signaling, such as cyclic nucleotide phosphodiesterase (PDE)-4 inhibitors, augment the ability of these approved therapies to induce differentiation in acute promyelocytic leukemia blast cells (Lerner and Epstein 2006). Efforts to identify others and potentially more effective differentiation inducers for the treatment of leukemia have remained a focus of major interest.

Cyclic AMP was the first second messenger reported, and since then numerous studies have shown its participation in many physiological and/or pathophysiological processes including cell cycle regulation. The signaling pathway mediated by this cyclic nucleotide has emerged as a key regulator of blood cell proliferation, differentiation, and apoptosis in malignant cell populations (Kobsar et al. 2008).

Cyclic AMP-elevating agents, including histamine H_2 agonists, are able to induce granulocyte differentiation in the human promyelocytic cell line HL-60 (Chaplinski and Niedel 1982; Nonaka et al. 1992). In M1 mouse myeloid leukemia cells as well as in the human promonocytic leukemia U937 cell line, dibutyryl cAMP (db-cAMP) but not H_2 agonists induces cell maturation (Honma et al. 1978; Shayo et al. 1997). In this regard, it was demonstrated the important role of the kinetic of the cAMP signaling in U937 cell differentiation (Lemos Legnazzi et al. 2000; Shayo et al. 2004). Interestingly, cAMP can also potentiate granulocytic differentiation of ATRA- or arsenic trioxide-induced maturation of human APL cells (Zhu et al. 2002; Guillemin et al. 2002; Nguyen et al. 2013).

Despite diverse extracellular signals activate GPCRs leading to an increase in cAMP, signal specificity results from accurate adjustments at different levels of the

cAMP-dependent pathway. Although cAMP is increased following H_2 receptor stimulation, in some leukemic cells differentiation fails to occur due to rapid receptor desensitization. Recently, cAMP efflux across MRP transporters was described in several systems as a regulator of intracellular cAMP levels modulating biological responses (Osycka-Salut et al. 2014; Copsel et al. 2014; Ventimiglia et al. 2015; Decouture et al. 2015). Both desensitization and extrusion processes will be discussed below.

However, it is important to consider recent reports indicating that cAMP can promote AML progression and protect myeloid leukemia cells against anthracycline- and arsenic trioxide-induced apoptosis (Gausdal et al. 2013; Safa et al. 2014). This suggests that the beneficial pro-differentiating and non-beneficial pro-survival effects of cAMP should be weighed against each other.

3.2.1 H_2 Receptor Desensitization Process as Pharmacological Target

Cyclic AMP is generated following the interaction of ligands with a receptor coupled to a transducer G protein. The occupied receptor promotes the exchange of GTP in the transducer, thus generating an activated subunit, which in turn activates the effector adenylyl cyclase (Marinissen and Gutkind 2001). The activation of this membrane signal transduction machinery is transient because several mechanisms are activated to terminate the stimulation and to return the cell to a resting state. These include the phosphorylation of the receptor by different kinases and the recruitment of β-arrestins, or inactivation of Gs via hydrolysis of GTP at a rate controlled by the regulators of G-protein signaling (RGS) protein (Freedman and Lefkowitz 1996). Activation of phosphodiesterases (PDEs) that are downstream of receptor/G-protein/effector coupling is an additional regulatory mechanism that induces the termination of the stimulus distal to the generation of cAMP (Conti et al. 1991).

Knowing that intracellular cAMP levels are important for leukemic differentiation, it is reasonable to assume that by targeting the mechanisms that regulate its intracellular levels, it would be possible to influence the ability of leukemic cells to be differentiated. In this sense, a proof of concept was to stably overexpress H_2 receptor to induce leukemic cell differentiation. In U937 cells, H_2 receptor overexpression triggered several mechanisms (namely, PDE activity induction and GRK-2 overexpression) tending to restore cAMP basal levels comparable to those of the naïve cells. The results obtained in time-course, dose-response, and desensitization experiments suggest that the mechanisms elicited as a consequence of receptor overexpression are able to manage cAMP basal levels but are not able to handle cAMP levels in stimulated conditions.

In spite of the onset of these regulatory mechanisms, the higher and sustained increase of cAMP levels caused by H_2 agonists in H_2 receptor overexpressing U937 cells induces differentiation and hampers the proliferation of the overexpression clone (Monczor et al. 2006). These findings provide new insights into the relevant role of receptor stoichiometry in the effector regulation on cell behavior and further suggest that this regulation may be externally manipulated to achieve beneficial therapeutic effects in the future.

There are seven members of the GRK family: GRK-1 through GRK-7. On the basis of sequence homology, these can be classified into three groups: GRK-1 (also known as rhodopsin kinase), GRK-2 and GRK-3 (also called β-adrenergic receptor kinases 1 and 2), and finally GRK-4, GRK-5, GRK-6, and GRK-7. The mechanisms by which GRK activity is regulated can be divided into three categories: subcellular localization, alterations in intrinsic kinase activity, and alterations in GRK expression levels. Cytosolic GRK-2 and GRK-3 are translocated to the membrane after receptor activation, in a process facilitated by the interaction with released Gβγ dimers (Palczewski 1997; Penn et al. 2000). Although GRK-2, GRK-3, GRK-5, GRK-6, and GRK-7 subtypes are ubiquitous, GRK-2 is particularly abundant in peripheral blood leukocytes and in myeloid and lymphoid cell lines (Chuang et al. 1992). GRK expression is tightly regulated and can be altered by different extracellular factors (Penela et al. 2003). It has also been demonstrated that their expression can be modified as a compensatory mechanism when the expression of one member is modified (Fernandez et al. 2007).

In U-937 leukemic cell line, the decrease in GRK-2 expression correlates with an increase of cAMP levels in response to different doses of H_2 agonist, in time-course cAMP accumulation experiments, and in desensitization assays. Hence, the reduction in GRK-2 expression determined a higher and prolonged cAMP response mediated by H_2 ligands allowing leukemic cell differentiation upon H_2 agonist treatment. These results establish an important correlation between duration and intensity of a signal and cellular response, showing that as a consequence of modulating the desensitization process, cells are able to switch from proliferation to differentiation pathway (Fernández et al. 2002). Overall, it can be concluded that GRK-2 plays a fundamental role modulating H_2 receptor signaling and that this kinase is to be considered a pharmacological target that, when intervened, is able to determine cell differentiation.

Structurally, GRK-2 protein contains an N-terminal RGS-homology domain (RH), a catalytic central domain (Kin), and a C-terminal region responsible for membrane localization (Penela et al. 2003). More recent experiments showed that the RGS domain and not kinase activity is necessary for H_2 receptor desensitization (Fernandez et al. 2011). This dual role of GRK-2 involving both functional domains (Kin and RH) is depicted in Fig. 2.

3.2.2 Cyclic AMP Efflux Mediated by MRP4 as a Target in Acute Myeloid Leukemia

Multidrug resistance protein 4 (MRP4) belongs to the C-branch of the superfamily of ATP-binding cassette transporters (ABC, ABCC4). These transporters are capable of actively pumping a wide range of endogenous and xenobiotic substrates out of the cells (Deeley et al. 2006). In particular, MRP4 has the ability to transport a broad variety of drugs including antivirals (adefovir, ganciclovir, tenofovir), antibiotics (cephalosporins), cardiovascular (thiazides, furosemide), and chemotherapeutic (methotrexate, 6-mercaptopurine, 6-thioguanine, topotecan) (Russel et al. 2008). However, the pathophysiological actions of these proteins are quite diverse, and transport of cytotoxic xenobiotics as a defense mechanism appears not

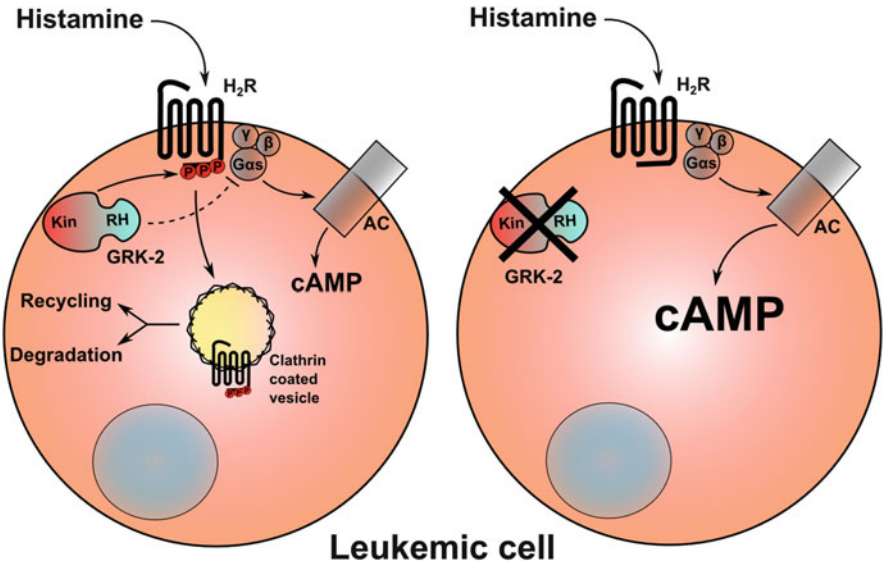

Fig. 2 Dual regulation of H2 receptor signaling by GRK-2. In the *left panel*, GRK-2 is able to regulate H2 receptor signaling through the activity of two functional domains. While the domain with kinase activity (Kin) phosphorylates the receptor in its C-term tail (*circled* Ps) inducing receptor internalization and recycling, the RGS-homology domain (RH) directly interacts with the G-protein inhibiting its activity. As mentioned in the main text, both processes modulate cAMP receptor signaling and make of GRK-2 a suitable target for inducing leukemic cell differentiation. In the right panel, as a consequence of GRK-2 downregulation or inhibition, H2 receptor signaling is increased and sustained over the time

to be the only important evolutionarily conserved function. Moreover, while several members of the ABC family are established as drug transporters, others also mediate transport of endogenous molecules. Indeed, MRP4 is capable of transporting a wide range of endogenous and signaling molecules including folates, bile acids, conjugated steroids, purine analogs, eicosanoids (prostaglandin E2, thromboxane TXB2, and leukotriene B4), ADP, and cyclic and nucleotides (cAMP and cGMP) (Russel et al. 2008). Remarkably, MRP4 is the major cAMP efflux transporter, and as already said, this cyclic nucleotide is involved in the regulation of cellular proliferation, differentiation, and apoptosis (Karin 1994). Recently, MRP4 mRNA and protein expression were found to be regulated by cAMP in Hela cells, vascular smooth muscle cells, megaloblastic leukemia M70e cells, and pancreatic adenocarcinoma cell lines (Bröderdorf et al. 2014; Carozzo et al. 2015). MRP4 expression is regulated through a mechanism where the balance between intracellular and extracellular cAMP plays a key role in the feedback regulation of the transporter expression. Persistent cAMP intracellular levels induce MRP4 promoter through the exchange proteins directly activated by cAMP (EPAC)/Rap1 pathway, whereas extracellular cAMP inhibits it through ERK phosphorylation (Carozzo et al. 2015).

Aside from physiological expression in blood cells, MRP4 has also been found in human leukemia cell lines (Oevermann et al. 2009; Copsel et al. 2011; Takeuchi et al. 2012). As in vitro it was clearly demonstrated that MRP4 confers resistance to nucleoside analog drugs and promotes the efflux of cyclic nucleotides, it has the potential to affect leukemia development and treatment. Therefore, in the last decade, the potential clinical relevance of this transporter has been specially examined in patients with AML. A clinical study for adult AML revealed the expression of MRP4 in blast cells with significant variability. Higher protein levels of this transporter were detected in the less differentiated FAB subtypes M0 and M1; however, its expression has no influence on treatment outcome using cytarabine. Furthermore, MRP4 expression did not correlate with remission rate and overall and relapse-free survival (Guo et al. 2009). On the contrary, a phase II clinical study in adult patients with AML in first relapse treated with gemcitabine and mitoxantrona revealed that higher expression of MRP4 and solute carrier family 29 member A2 correlated with not achieving complete remission (Advani et al. 2010).

When 53 children with de novo AML were evaluated, MRP4 mRNA expression was found in all patients. Nevertheless, as in adult AML, MRP4 in childhood AML was not associated with the failure to achieve remission (Steinbach et al. 2003). Recently, frequent copy number alterations of MRP4 were observed in de novo AML, and variable expression of this transporter was detected among AML subtypes from 155 pediatric patients. Although some authors found the highest levels of MRP4 in the less differentiated AML subtypes, in this study, MRP4 expression was found to be higher in the M7 AML subtype (Lian et al. 2013).

As MRP4 is the major cAMP efflux transporter, current evidences suggest that MRP4 is implicated not only in chemotherapy resistance but also in cancer biology. Indeed, the mere genetic silencing or pharmacologic inhibition of MRP4 reduced tumor growth in a xenograft AML model. Furthermore, MRP4 knockdown induced cell cycle arrest and apoptosis in vivo (Copsel et al. 2014). As it was mentioned above, the finding that MRP4 overexpression confers nucleoside analog drugs resistance has strong implications for leukemia chemotherapy (Adachi et al. 2002).

In particular, MRP4 expression was detected in KG-1, HL-60, U937, KG-1a, and AML cell lines, and its expression decreases during leukocyte differentiation promoting cAMP accumulation in differentiated cells (Oevermann et al. 2009; Takeuchi et al. 2012). In accordance, it was demonstrated that besides playing a role in drug-resistant leukemia cell lines, MRP4 regulates leukemia cell proliferation and differentiation through the endogenous MRP4 substrate, cAMP (Copsel et al. 2011). The signaling pathway mediated by this cyclic nucleotide has emerged as a key regulator of blood cell proliferation, differentiation, and apoptosis in malignant cell populations (Kobsar et al. 2008). Thus, H_2 agonist when combined with MRP4 and PDE4 inhibitors induces cell cycle arrest and maturation in U937 cells. By using two well-characterized MRP inhibitors such as probenecid and MK571 in intact cells and membrane vesicles, it has been shown that MRP inhibition further enhanced H_2 receptor-induced intracellular cAMP concentration, allowing cell growth inhibition and differentiation. MRP pharmacological

inhibition or knockdown modified the intracellular content of cAMP concomitantly with an accentuated decrease in the proliferative rate of U937 cells. This inhibition was even more pronounced when MRP inhibitors were combined with cAMP-stimulating agents, such as H_2 receptor agonists (Copsel et al. 2011; Werner et al. 2015).

Altogether these findings indicate that agents that modulate or mimic cAMP levels should be considered as a new alternative strategy for AML treatment, either alone or in combination with chemotherapeutic drugs.

4 Final Considerations

Histamine, as a wide distributed local mediator and neurotransmitter, mediates many cell functions and its receptors are potential targets for the treatment of several diseases. Hematopoietic cells express histamine receptors, and their modulation has the potential to ameliorate their pathologies. Among years, histamine ligands prove to be of clinical utility and are among the top marketed drugs around the world. This did not prevent the search and finding of novel therapeutic uses, providing promising results concerning cancer treatment, specifically involving AML. Up to now, two main strategies have been pursued: the complementation of chemotherapeutics to allow immune rejection of cancer cells in a graft-versus-host type of reaction and the induction of differentiation of malignant cells to eliminate abnormal cell proliferation and to induce terminal differentiation recovering the functionality of the original tissue. Thus, the treatment with histamine or H_2 agonists in combination with IL-2 or GRK2, PDE4, or MRP4 inhibitors represents a therapeutic scheme with great potentiality. The results obtained in preclinical and clinical studies grant further research to achieve optimized treatments with fewer side effects.

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Histamine H_1 Receptor Gene Expression and Drug Action of Antihistamines

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Abstract

The upregulation mechanism of histamine H_1 receptor through the activation of protein kinase C-δ (PKCδ) and the receptor gene expression was discovered. Levels of histamine H_1 receptor mRNA and IL-4 mRNA in nasal mucosa were elevated by the provocation of nasal hypersensitivity model rats. Pretreatment with antihistamines suppressed the elevation of mRNA levels. Scores of nasal symptoms were correlatively alleviated to the suppression level of mRNAs above. A correlation between scores of nasal symptoms and levels of histamine H_1 receptor mRNA in the nasal mucosa was observed in patients with pollinosis. Both scores of nasal symptoms and the level of histamine H_1 receptor mRNA were improved by prophylactic treatment of antihistamines. Similar to the antihistamines, pretreatment with antiallergic natural medicines showed alleviation of nasal symptoms with correlative suppression of gene expression in nasal hypersensitivity model rats through the suppression of PKCδ. Similar effects of antihistamines and antiallergic natural medicines support that histamine H_1 receptor-mediated activation of histamine H_1 receptor gene expression is an important signaling pathway for the symptoms of allergic diseases. Antihistamines with inverse agonist activity showed the suppression of constitutive histamine H_1 receptor gene expression, suggesting the advantage of therapeutic effect.

Keywords

Allergic disease-sensitive gene • Histamine H_1 receptor • Natural medicine • Pollinosis • Protein kinase C

Abbreviations

GPCR	G-protein-coupled receptor
HDC	Histidine decarboxylase
H1R	Histamine H_1 receptor
IL-5	Interleukin-5
PARP-1	Poly(ADP-ribose) polymerase-1
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
TDI	Toluene 2,4-diisocyanate

1 Introduction

Histamine is a key mediator of immune hypersensitivity (type-I allergy), and the histamine H_1 receptor mediates major symptoms of allergic diseases. Thus, antihistamines are the first therapeutic choice for allergic diseases. Although antihistamines target the histamine H_1 receptor, the mechanism of their therapeutic action remains unknown.

Histamine H_1 receptor is a G-protein-coupled receptor (GPCR) with seven transmembrane spanning domains (Yamashita et al. 1991; Shimamura et al. 2011). Histamine H_1 receptor desensitization is a well-known mechanism to reduce histamine H_1 receptor signaling, and the receptor downregulation is the final step of the receptor desensitization. In contrast, the mechanism of histamine H_1 receptor upregulation was discovered by the stimulation of histamine H_1 receptor through the activation of the receptor gene (Das et al. 2007). The upregulation of histamine H_1 receptor resulted in the increase of receptor signaling (Ohuchi et al. 1998). Histamine H_1 receptor upregulation in patients with allergic rhinitis was reported (Iriyoshi et al. 1996; Dinh et al. 2005). Then it was hypothesized that upregulation of histamine H_1 receptor exacerbates the symptoms of allergic diseases where histamine H_1 receptor gene works as an allergic disease-sensitive gene.

In the present review, molecular mechanism of histamine H_1 receptor gene expression is described. Then alleviation of nasal symptoms and correlative suppression of histamine H_1 receptor gene expression by antihistamines are described for their clinical significance. In addition, alleviation of nasal symptoms and correlative suppression of histamine H_1 receptor gene expression by antiallergic natural medicines and pharmacological mechanism of active compounds from natural medicines at the molecular level are also described in order to define the importance of histamine H_1 receptor gene expression for the symptoms of allergic diseases.

2 Intracellular Signaling for Histamine H_1 Receptor Gene Expression

Histamine H_1 receptor downregulation, which is thought as the final step of desensitization, was induced after the stimulation of histamine H_1 receptor. The downregulation was dependent on ubiquitin/proteasome system following clathrin-mediated internalization (Hishinuma et al. 2010). Phosphorylation of five amino acid residues, three threonine residues (Thr^{140}, Thr^{142}, and Thr^{478}), and two serine residues (Ser^{396} and Ser^{398}) in the intercellular domains was suggested to participate the downregulation. Protein kinases such as protein kinase A, protein kinase C, protein kinase G, and calcium calmodulin-dependent protein kinase II showed the phosphorylation activity of five amino acid residues (Horio et al. 2004).

On the other hand, stimulation of the histamine H_1 receptor on HeLa cells induced histamine H_1 receptor upregulation (Das et al. 2007). Increases in

histamine H_1 receptor mRNA and histamine H_1 receptor promoter activity preceded to the upregulation.

Stimulation of histamine H_1 receptor activates and phosphorylates PKCδ and translocates PKCδ to Golgi (Mizuguchi et al. 2011a). Activations of ERK and poly (ADP-ribose) polymerase-1 (PARP-1) are followed to elevate histamine H_1 receptor gene expression. Several binging sites for control elements were located in the prompter region of histamine H_1 receptor gene and separated into two regions (Mizuguchi et al. 2012). Sites for two AP-1 and one Ets-1 were located in the upstream region, and one Ku86/Ku70 was in the downstream region. Ku86 was responsible for DNA binding and poly(ADP-ribosyl)ated in response to PKCδ signaling, inducing its dissociation from the downstream region that is crucial for promoter activity.

3 Histamine H_1 Receptor Gene Expression in Nasal Hypersensitivity Model Rats

Nasal hypersensitivity model rats sensitized with toluene 2,4-diisocyanate (TDI) are useful for the study of nasal hypersensitivity (Kitamura et al. 2004). Upregulations of histamine H_1 receptor and its mRNA were induced in the nasal mucosa by the provocation of nasal hypersensitivity with TDI. Upregulation of histamine H_1 receptor mRNA was partially suppressed by the short-term treatment (less than 3 days) of antihistamines and almost completely suppressed by the long-term treatment (longer than 1 week) (Mizuguchi et al. 2008). In spite of almost all suppression of histamine H_1 receptor mRNA upregulation, symptoms of nasal hypersensitivity were only partially alleviated. The data suggests the existence of the second nasal hypersensitivity-sensitive gene.

4 Histamine H_1 Receptor Gene Expression in Pollinosis Patients

Correlation between symptoms of nasal hypersensitivity and histamine H_1 receptor mRNA level in nasal mucosa was observed in patients with pollinosis by the clinical study (Mizuguchi et al. 2010). Prophylactic treatment of antihistamines alleviated nasal symptoms and suppressed histamine H_1 receptor mRNA level. In another study by controlled exposure to pollen using an environmental exposure unit, upregulation of histamine H_1 receptor mRNA level in nasal mucosa of patients with pollinosis was suppressed by the prophylactic treatment of antihistamines, and nasal symptoms were correlatively alleviated (Kitamura et al. 2015). The data strongly suggest that histamine H_1 receptor gene is an allergic disease-sensitive gene.

5 Antihistamines with Inverse Agonist Activity

Several antihistamines such as cetirizine, loratadine, and epinastine showed inverse agonist activity (Leurs et al. 2002), and NF-kB signaling was inhibited by antihistamines with inverse agonist activity (Bakker et al. 2002; Wu et al. 2004). Antihistamines with inverse agonist activity showed constitutive histamine H_1 receptor gene expression which plays an important role in symptoms of allergic diseases (Mizuguchi et al. 2011b, 2013).

6 Pharmacological Mechanism of Active Compounds from Antiallergic Natural Medicines on Histamine H_1 Receptor-Mediated Histamine H_1 Receptor Gene Expression

Sho-seiryu-to is a representative formula of *Kampo* medicine for the therapy of allergic diseases. Similar to the effect of antihistamines, partial alleviation of nasal symptoms was observed by the long-term pretreatment of *Sho-seiryu-to* in the nasal mucosa of TDI-sensitized nasal hypersensitivity model rats (Das et al. 2009). Correlation between symptoms and mRNA levels of histamine H_1 receptor and IL-4 was observed. However, alleviation of symptoms were partial in spite of almost complete suppression of histamine H_1 receptor mRNA upregulation. Similar results were obtained when *Kujin* (*Sophora flavescens*), another antiallergic *Kampo* medicine; *Tephrosia purpurea*, an antiallergic *Ayurvedic* medicine; and quercetin, a compound from traditional antiallergic foods, were treated (Dev et al. 2009; Shill et al. 2015; Hattori et al. 2013). (-)Maackiain and 4-methoxybenzofuran-5-carboxamide were successfully identified as suppressing compounds of histamine H_1 receptor and IL-4 gene expression from *Kujin* (*S. flavescens*) and *T. purpurea*, respectively, and showed partial alleviation of symptoms, similar to their original natural medicines.

PKCδ, a key signal molecule of histamine H_1 receptor gene expression, revealed to be the site of pharmacological action of (-)maackiain, 4-methoxybenzofuran-5-carboxamide, and quercetin (Mizuguchi et al. 2015; Shill et al. 2015; Hattori et al. 2013). The data strongly suggest that the target molecule of antiallergic natural medicines acts as a common site of action in histamine H_1 receptor gene expression mechanism. Histamine H_1 receptor/PKCδ/MEK/ERK/poly (ADP-ribose) polymerase-1 (PARP-1)-mediated activation of histamine H_1 receptor gene expression, which is suppressed by antihistamines at histamine H_1 receptor level and antiallergic natural medicines at PKCδ level, is suggested to be an important signal for symptoms of allergic diseases.

Heat shock protein 90 (Hsp90) revealed to be a direct target of (-)maackiain (Nariai et al. 2015). The mechanism is suggested that histamine H_1 receptor signaling induces the translocation of Hsp90-PKCδ complex from cytosol to Golgi through the activation of PKCδ. Binding of (-)maackiain to Hsp90 is suggested to suppress histamine H_1 receptor gene expression by disrupting Hsp90-PKCδ complex (Figs. 1 and 2).

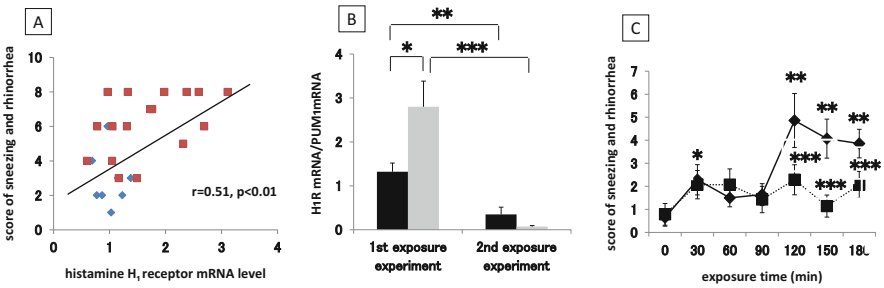

Fig. 1 (**a**) Correlation between scores of nasal symptoms and histamine H_1 receptor mRNA level in the nasal mucosa of patients with pollinosis. (Filled square) Non-prophylactic treatment and (filled diamond) prophylactic treatment with antihistamines. (**b**) Effects of the first pollen exposure without treatment and the second exposure after prophylactic administration of antihistamine for 3 days on the levels of H1R mRNA in nasal mucosa of the 14 responder patients with pollinosis. Black columns: pre-pollen exposure (first or second). *Gray* columns: post-pollen exposure (first or second). $*P < 0.01$, $**P < 0.01$ vs. pre-first pollen exposure. $***P < 0.01$ vs. post-first pollen exposure. $n = 14$. (**c**) Effects of the first pollen exposure without treatment and the second exposure after prophylactic administration of antihistamine for 3 days on the sum of sneezing and rhinorrhea scores in the 14 responder patients with pollinosis. (Filled diamond) First exposure without treatment; (filled square) second exposure after prophylactic administration of ebastine for 3 days. $*P < 0.05$, $**P < 0.01$ vs. pre-first exposure, $**P < 0.01$ vs. first exposure. $n = 14$

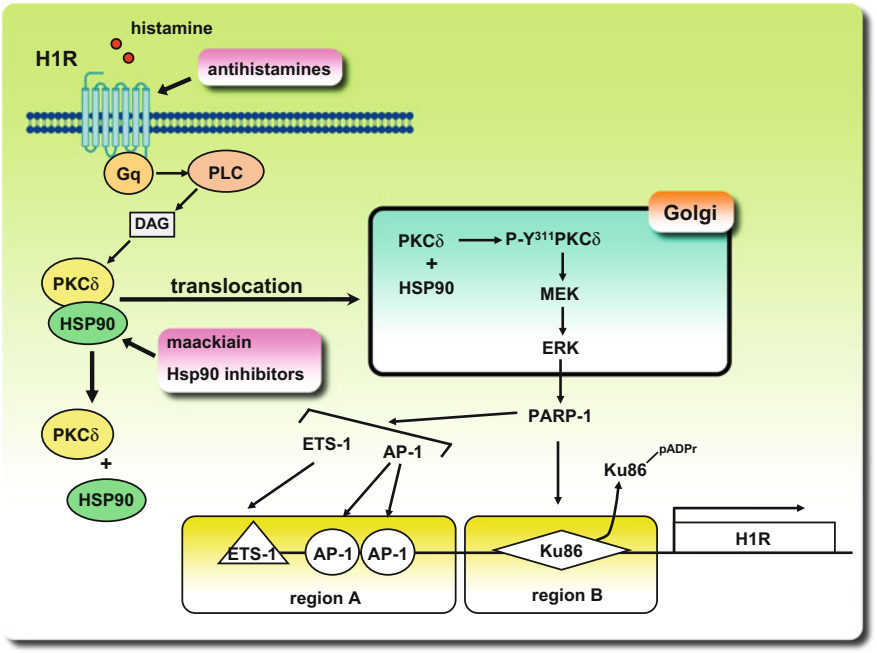

Fig. 2 Schema of histamine H_1 receptor-mediated upregulation of histamine H_1 receptor through PKCδ activation-mediated histamine H_1 receptor gene expression

7 Conclusion

Histamine H_1 receptor stimulation induced histamine H_1 receptor upregulation through PKCδ/MEK/ERK/poly(ADP-ribose) polymerase-1 (PARP-1) signaling-mediated activation of histamine H_1 receptor gene expression. Suppression of histamine H_1 receptor mRNA upregulation and alleviation of nasal symptoms were correlatively induced by the long-term treatment of antihistamines both in nasal hypersensitivity model rats and in patients with pollinosis. Antihistamines and antiallergic natural medicines showed similar correlative effects on alleviation of nasal symptoms and suppression of histamine H_1 receptor mRNA upregulation. The data suggests histamine H_1 receptor gene is an allergic disease-sensitive gene, and the activation of histamine H_1 receptor gene is suppressed at the different sites of signaling. Histamine H_1 receptor gene was suggested to form a group of allergic disease-sensitive gene with genes of HDC, IL-5, and IL-4. Antihistamines may have an advantage of therapeutic effect by stronger suppression of histamine H_1 receptor gene expression.

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Histamine Clearance Through Polyspecific Transporters in the Brain

Takeo Yoshikawa and Kazuhiko Yanai

Contents

Abstract

Histamine plays an important role as a neurotransmitter in diverse brain functions, and clearance of histamine is essential to avoid excessive histaminergic neuronal activity. Histamine *N*-methyltransferase, which is an enzyme in the central nervous system that metabolizes histamine, is localized to the cytosol. This suggests that a histamine transport process is essential to inactivate histamine. Previous reports have shown the importance of astrocytes for histamine transport, although neuronal histamine transport could not be ruled out. High-affinity and selective histamine transporters have not yet been discovered, although it has been reported that the following three polyspecific transporters transport histamine: organic cation transporter (OCT) 2, OCT3, and plasma membrane monoamine transporter (PMAT). The K_m values of human OCT2, OCT3, and PMAT are 0.54, 0.64, and 4.4 mM, respectively. The three transporters are expressed in the brain, and their regional distribution is different.

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Recent studies revealed the contribution of OCT3 and PMAT to histamine transport by primary human astrocytes. Several investigations using mice supported the importance of OCT3 for histamine clearance in the brain. However, further studies are required to elucidate the detailed mechanism of histamine transport in the brain.

Keywords
Diamine oxidase • Histamine • Histamine *N*-methyltransferase • Organic cation transporter 2 • Organic cation transporter 3 • Plasma membrane monoamine transporter • Polyspecific transporter

1 Introduction

Histamine plays an important role as a neurotransmitter in the human brain. Histamine neurons project their axons from the tuberomammillary nucleus of the posterior hypothalamus to the entire brain (Watanabe et al. 1984). Brain histamine plays a role in diverse physiological functions such as anxiety, sleep-wake cycles, appetite, and learning and memory (Haas et al. 2008). Dysfunction of the histaminergic nervous system is associated with various brain diseases, including eating disorders (Yoshizawa et al. 2009), Tourette's syndrome (Ercan-Sencicek et al. 2010), and depression (Yanai and Tashiro 2007). In addition, decreased histamine concentration has been found in the cerebrospinal fluid of narcolepsy patients (Nishino et al. 2009), and neuronal histamine deficit was observed in Alzheimer's disease (Panula et al. 1998). Thus, a reduced histamine concentration may contribute to the pathogenesis of various neuropsychiatric disorders.

Neurotransmitter clearance is very important in the maintenance of normal neurotransmitter concentrations. Neurotransmitters released into the synaptic cleft are immediately inactivated by enzymes and/or moved through transporters in neurons or adjacent glial cells. These mechanisms are extremely important in preventing excessive neuronal activity. This is evident from the fact that various neurological disorders, including depression and schizophrenia, have been caused by dysfunctional neurotransmitter clearance. Thus, many drugs that modulate neurotransmitter clearance, including selective serotonin reuptake inhibitors and monoamine oxidase inhibitors, have been developed and extensively used in patients suffering from brain diseases. In terms of the histaminergic nervous system, extensive studies have identified important enzymes and transporters involved in histamine clearance, although the complete mechanism of histamine clearance remains unknown.

2 Histamine-Metabolizing Enzymes

Histamine is metabolized by two different enzymes, diamine oxidase (DAO; E.C 1.4.3.6) and histamine *N*-methyltransferase (HNMT; EC 2.1.1.8) (Maintz and Novak 2007) (Fig. 1). Human DAO is a homodimeric protein composed of two 85 kDa subunits and catalyzes the oxidative deamination of histamine (McGrath et al. 2009). DAO is mainly expressed in intestine and kidney and inactivates histamine in peripheral tissues (Hesterberg et al. 1984). However, DAO expression and activity in brain are low or absent (Klocker et al. 2005), indicating negligible involvement of DAO in brain histamine clearance. HNMT, a 33 kDa protein, is expressed in many organs including the brain. HNMT catalyzes the methylation of histamine in the presence of S-adenosylmethionine (Horton et al. 2001), and histamine receptors are inactive to the resulting metabolite *tele*-methylhistamine (tMH). A single administration of metoprine, which is an HNMT inhibitor, reportedly increased mouse brain histamine concentration (Kitanaka et al. 2007). We have confirmed that the histamine concentration in brain lysates of HNMT-deficient mice was significantly higher than that of wild-type mice (unpublished observation). Therefore, HNMT plays an essential role in brain histamine inactivation.

HNMT has been recognized as a cytosolic protein since high HNMT activity was reported in soluble fractions extracted from brains (Brown et al. 1959). The cytosolic distribution of HNMT was later confirmed by Nishibori et al. (Nishibori et al. 2000). However, Barnes and Hough reported that mouse HNMT existed in the plasma membrane and membrane-bound HNMT inactivated extracellular histamine (Barnes and Hough 2002). Although the molecular identity of HNMT as a membrane protein remains unclear, HNMT has one hydrophobic amino acid region (amino acid region between F158 and W180). However, this region played a crucial role in methyltransferase activity (Horton et al. 2001), indicating that HNMT activity would be lost if the region was a transmembrane domain. Ogasawara et al. reported that HNMT could translocate to membrane under growth factor stimulation, though the membrane-associated form of HNMT had quite low enzymatic activity (Ogasawara et al. 2006b). We recently used subcellular fractionation and confocal microscope observations to demonstrate that human HNMT is a cytosolic protein and not a membrane protein (Yoshikawa et al. 2013). These lines of evidence indicate that HNMT is a cytosolic protein that inactivates intracellular histamine, emphasizing that histamine requires trafficking into the cytosol via transporters, where it is then subsequently metabolized to inactive tMH by HNMT.

3 Contribution of Astrocytes to Histamine Transport in Brain

Neurotransmitters released from a presynapse are removed from synaptic cleft by neurons and/or astrocytes. Monoamine transporters, such as those for norepinephrine and serotonin, are expressed in presynaptic neurons (Torres et al. 2003). Amino

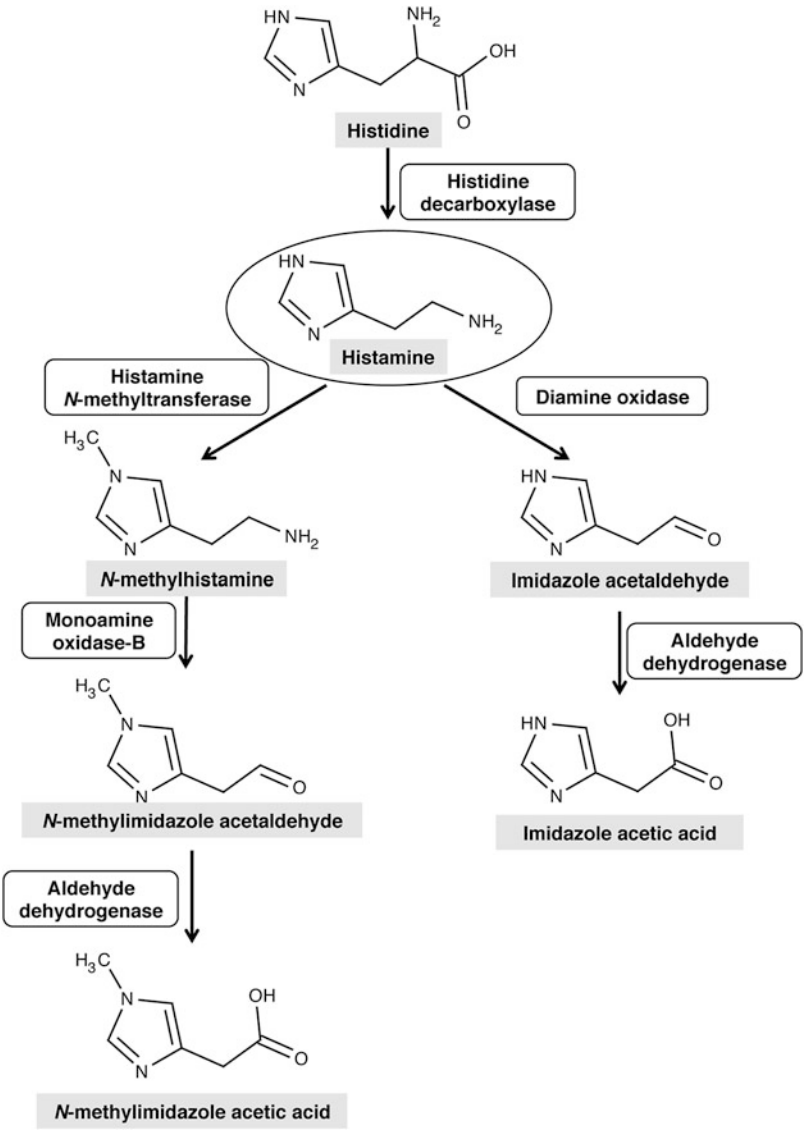

Fig. 1 Histamine synthesis and degradation. Histamine synthesis from L-histidine is catalyzed by histidine decarboxylase. Histamine *N*-methyltransferase inactivates histamine by adding methyl group at the imidazole nitrogen in the presence of S-adenosylmethionine. The resultant *N*-methylhistamine is further metabolized by monoamine oxidase-B (Lin et al. 1993) and aldehyde dehydrogenase (Ambroziak and Pietruszko 1987). Oxidative deamination by diamine oxidase is another pathway to metabolize histamine

acid neurotransmitters such as glutamate and GABA are usually taken up into both adjacent astrocytes and neurons (Zhou and Danbolt 2013). Previous studies investigated whether neurons or astrocytes are involved in histamine transport. Rafalowska et al. reported that astrocytes, but not synaptosomes, were capable of histamine uptake (Rafalowska et al. 1987). Huszti et al. examined the effect of astrocyte-specific toxins such as alpha-aminoadipic acid and fluoroacetate on histamine transport. Inactivation of astrocytes by these reagents significantly reduced the histamine transport activity in vitro (Huszti et al. 1994) and in vivo (Huszti et al. 1998), emphasizing the importance of astrocytes for histamine transport. However, several groups reported neuronal localization of HNMT (Nishibori et al. 2000; Shan et al. 2012), and our group showed the presence of histamine uptake into rat synaptosomes (Sakurai et al. 2006). Further studies are necessary to reveal the importance of neurons for histamine clearance.

4 Polyspecific Transporters for Histamine Transport

Extracellular neurotransmitters are removed by two different uptake systems: uptake-1 and uptake-2. The uptake-1 system is characterized as a transport system with high-affinity (i.e., lower Michaelis constant (K_m) value), low-capacity (i.e., lower maximum transport velocity (V_{max}) value), and Na^+/Cl^--dependent transporters with high substrate specificity. For example, dopamine uptake through a dopamine transporter is classified as uptake-1 transport (Vaughan and Foster 2013). These neurotransmitter transporters on nerve end terminals play an important role in the removal of excessive neurotransmitters and in normal aminergic neurotransmission. Various psychoactive drugs regulating the uptake-1 transport system, such as serotonin-selective reuptake inhibitors, have been used for patients suffering from neurological diseases (Zarate et al. 2013). The uptake-2 system transports neurotransmitters through low-affinity (i.e., higher K_m value), high-capacity (higher V_{max} value), and Na^+/Cl^--independent transporters with low substrate specificity. The uptake-2 system was previously named as the extraneuronal monoamine transport system and was considered to work mainly in peripheral organs including the myocardial cells (Iversen 1965), placenta (Wu et al. 1998), and kidney (Motohashi and Inui 2013). However, in the last decade, extensive studies have demonstrated that the uptake-2 system also maintains aminergic neurotransmissions and is involved in diverse functions such as anxiety, stress response, and antidepressant efficacy (Courousse et al. 2015; Daws 2009; Daws et al. 2013; Horton et al. 2013; Matthaeus et al. 2015).

Although high-affinity and low-capacity histamine transporters belonging to the uptake-1 system have not been identified, histamine is known to be transported through the uptake-2 system. Currently, three low-affinity, high-capacity, and Na^+/Cl^--independent histamine transporters have been identified: organic cation transporter 2 (OCT2) (Grundemann et al. 1998a; Ogasawara et al. 2006a), OCT3 (Grundemann et al. 1998b), and plasma membrane monoamine transporter (PMAT) (Engel et al. 2004). Characteristics of these three transporters are

Table 1 Basic data of OCT2, OCT3, and PMAT

	OCT2	OCT3	PMAT
Gene name	SLC22A2	SLC22A3	SLC29A4
Cloning year	1996 (rat)[a]	1998 (rat)[b]	2004 (human)[c]
Molecular weight	63 kDa	61 kDa	58 kDa
Amino acid	584 aa	556 aa	530 aa
Amino acid homology	Rat 83% Mouse 76%	Rat 85% Mouse 86%	Rat 86% Mouse 87%
Transmembrane domains	12	12	11
Expression in the peripheral tissues	Kidney[a]	Placenta, skeletal muscle, testis, lung, liver, kidney[b]	Heart, adrenal gland, small intestine[c]

[a]Okuda et al. (1996); [b]Kekuda et al. (1998); [c]Engel et al. (2004)

summarized in Tables 1 and 2. High K_m values of these polyspecific transporters to histamine might be misinterpreted as lack of their transport ability. However, K_m value alone is not a reliable factor to determine the transport efficiency (the rate which a substrate is trafficking through a transporter per unit time). V_{max}/K_m ratio is recognized as a useful indicator to evaluate the transport efficiency (Schomig et al. 2006). V_{max} values of polyspecific monoamine transporters are 2–3 orders of magnitude higher than those of uptake-1 system, indicating that V_{max}/K_m of uptake-2 system is roughly comparable to that of uptake-1 system.

Synaptic concentration of serotonin in the brain is estimated to be around 6 mM (Bunin and Wightman 1998). Lim et al. reported that the pK_i value of human H1 receptor was 4.2 (Lim et al. 2005). These results suggest that brain histamine concentration might be over 50 μM after stimulation, although histamine concentration in the brain is not determined until now. The K_m values of uptake-1 system to catecholamines are usually less than 5 μM, implying that polyspecific monoamine transporters are suited for histamine clearance in the brain.

5 Organic Cation Transporter 2

OCT2, which is encoded by *Slc22a2* gene, was discovered from rat kidney in 1996 (Okuda et al. 1996). Human OCT2, first cloned in 1997, is a 63 kDa protein composed of 584 amino acids (Gorboulev et al. 1997) and is expressed mainly in the kidney (Motohashi et al. 2002). Substrates for human OCT2 are the model cations such as tetraethylammonium and 1-methyl-4-phenylpyridinium (MPP) and various drugs including histamine H2 receptor antagonists. Human OCT2 also transports neurotransmitters: acetylcholine, dopamine, norepinephrine, serotonin, and histamine (Koepsell 2013). The transport activity of human OCT2 is decreased by phosphatidylinositol 3-kinase (PI3K) and protein kinase A (PKA) and is potentiated by Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (Cetinkaya

Table 2 Pharmacological characteristics of OCT2, OCT3, and PMAT

	OCT2	OCT3	PMAT
K_m value to histamine Human Rat Mouse	1.3[a], 0.94[b] mM 0.89 mM[b] 111 μM[c]	0.22 [b], 0.64[d] mM 0.54 mM[b] 1.6 mM[c]	4.4 mM[d] 1.5 mM[c]
K_m value to Dopamine Norepinephrine Serotonin	390 μM[a] 1.9 mM[a] 80 μM[a]	1.0 mM[d] 923 μM[d] 988 μM[d]	406 μM[d] 1.1 mM[d] 283 μM[d]
Distribution in brain	Cortex, hippocampus, thalamus, hypothalamus, dorsal raphe nucleus, locus coeruleus, etc.[a,b,e]	Cortex, hippocampus, midbrain, cerebellum, etc.[b,f]	Forebrain cortex, olfactory tubercle, hippocampus, cerebellum choroid plexus, etc.[g,h]
Regulation by kinases	PKA↓[i] PI3K↓[i] CaMKII↑[i]	CaMKII↑[j]	N.D.
IC_{50} of inhibitors Decynium-22 Corticosterone Tetraethylammonium Cimetidine Imipramine	1.13 μM[k] 34.2 μM[k] 76 μM (K_m)[l] 8.6 μM (K_m)[l] 3.9 μM[m]	0.09 μM[k] 0.29 μM[k] 1.37 mM[l] 42 μM[l]	0.10 μM[n] 450.5 μM[n] 8.8 mM (K_m)[n] 21.1 μM[o]
Physiological brain functions	Anxiety[p], Stress vulnerability[q]	Anxiety[r], dopaminergic degeneration[f]	Autism spectrum disorder[s]

PKA protein kinase A, *PI3K* phosphatidylinositol 3-kinase, *CaMKII* Ca^{2+}/calmodulin-dependent protein kinase II, IC_{50} half maximal inhibitory concentration

[a]Busch et al. (1998); [b]Amphoux et al. (2006); [c]our unpublished observation; [d]Duan and Wang (2010); [e]Bacq et al. (2012); [f]Cui et al. (2009); [g]Dahlin et al. (2007); [h]Vialou et al. (2007);[i] Cetinkaya et al. (2003); [j]Ciarimboli and Schlatter (2005); [k]Hayer-Zillgen et al. (2002); [l]Koepsell et al. (2003); [m]Belzer et al. (2013); [n]Engel et al. (2004); [o]Haenisch and Bonisch (2010); [p]Bacq et al. (2012); [q]Courousse et al. (2015); [r]Wultsch et al. (2009); [s]Adamsen et al. (2014)

et al. 2003). Although OCT2 expression in CNS is lower than that in the kidney, several reports showed the expression of OCT2 in the brain including the cortex, hippocampus, thalamus, hypothalamus, dorsal raphe nucleus, and locus coeruleus (Bacq et al. 2012; Busch et al. 1998; Courousse et al. 2015; Nakata et al. 2013). Particularly, immunoelectron microscopic analysis demonstrated that OCT2 was localized to synaptic vesicles in presynaptic terminals, indicating the involvement of this transporter in neurotransmitter clearance (Nakata et al. 2013). The disruption of mouse OCT2 induced the dysregulation of norepinephrine and serotonin concentration and changed the sensitivity to antidepressant drugs (Bacq et al. 2012). Courousse et al. also reported the involvement of OCT2 in stress vulnerability in mice (Courousse et al. 2015).

K_m values of OCT2 to histamine were 111 μM (mouse OCT2), 0.89 mM (rat OCT2), and 0.94 or 1.3 mM (human OCT2). Among monoamine neurotransmitters,

histamine was a preferable substrate for mouse (unpublished observation), rat, and human OCT2 (Amphoux et al. 2006), although the direct involvement of OCT2 in histamine transport has not been demonstrated. Perdan-Pirkmajer et al. reported that neonatal rat astrocytes showed histamine transport activity and expressed OCT2, suggesting the possible involvement of OCT2 in histamine transport by rat astrocytes. However, the K_m value of rat OCT2 for histamine was significantly higher than that of rat astrocytes, and Na^+-dependency on histamine transport was different between OCT2 and rat astrocytes (Perdan-Pirkmajer et al. 2013). Our group showed human OCT2 was not expressed in primary human astrocytes and human astrocytoma cell lines (Naganuma et al. 2014; Yoshikawa et al. 2013). These results indicated a low or absent contribution of OCT2 to astrocyte histamine transport. Several studies have revealed that OCT2 is dominantly expressed in neurons, so further studies are necessary to reveal the involvement of neuronal OCT2 in histamine clearance in the brain.

6 Organic Cation Transporter 3

In 1998 the rat and human OCT3 genes were cloned from rat placenta and the human kidney carcinoma cell line Caki-1 cells, respectively (Grundemann et al. 1998b; Kekuda et al. 1998). Human OCT3 is a 61 kDa protein composed of 556 amino acids and has 12 putative transmembrane domains. OCT3 is abundantly expressed in placenta and also in the intestine, heart, and brain. Human OCT3 is activated by CaMKII but not PKA and protein kinase C (PKC) (Ciarimboli and Schlatter 2005). OCT3 transports various neurotransmitters, including histamine, and is widely expressed in different brain regions such as the hippocampus, cerebellum, and cerebral cortex (Wu et al. 1998). Recent studies have revealed that brain OCT3 played an important role in the clearance of neurotransmitter, including serotonin (Baganz et al. 2008) and dopamine (Cui et al. 2009).

K_m values of OCT3 to histamine were 1.6 mM (mouse OCT3), 0.54 mM (rat OCT3), and 0.22 or 0.64 mM (human OCT3) (Amphoux et al. 2006; Duan and Wang 2010). We have already shown the expression of OCT3 in human astrocytes and the involvement of OCT3 in histamine transport by primary human astrocytes. OCT3 has also been expressed in mouse (Cui et al. 2009) and rat astrocytes (Takeda et al. 2002), suggesting the importance of OCT3 for histamine clearance. Gasser et al. showed the potential role of OCT3 in histamine clearance around hypothalamic area (Gasser et al. 2006). Zhu et al. demonstrated that mouse OCT3 was responsible for the clearance of ischemia-induced histamine (Zhu et al. 2012). Although in vitro pharmacological assays could not support the importance of OCT3 for histamine transport by primary rat astrocytes (Perdan-Pirkmajer et al. 2013), these lines of evidences strongly suggest the involvement of OCT3 in histamine transport.

7 Plasma Membrane Monoamine Transporter

PMAT encoded by *SLC29A4* gene was newly identified in 2004 (Engel et al. 2004). PMAT is a 58 kDa protein composed of 530 amino acids. Human *PMAT* mRNA was strongly expressed in the skeletal muscle and brain and moderately in the kidney, liver, and heart. PMAT transports MPP^+ and various neurotransmitters, with MPP^+ transport through human PMAT being independent on extracellular Na^+/Cl^- concentration. Residues of isoleucine 89 and tyrosine 112 are recognized as essential amino acids for transport activity (Ho and Wang 2010; Ho et al. 2012). PMAT, which is widely expressed in the brain (Dahlin et al. 2007; Vialou et al. 2007), has been shown to be involved in serotonin clearance (Daws et al. 2013; Matthaeus et al. 2015). Mutations in the PMAT gene are associated with autism spectrum disorder (Adamsen et al. 2014), although disruption of PMAT did not induce an overt phenotype in mice (Duan and Wang 2013). PMAT is expressed in the tuberomammillary nucleus in the posterior hypothalamus (Vialou et al. 2007), suggesting the possible involvement of PMAT in histamine transport. We showed that PMAT had a predominant role in histamine transport by primary human astrocytes. Rat astrocytes also expressed PMAT (Wu et al. 2015), although the importance of PMAT for histamine clearance by rat astrocytes was not determined.

8 Future Perspectives

We recently investigated the histamine transport mechanism by primary human astrocytes. Human astrocytes transported histamine in a time- and dose-dependent manner. Analysis of transport kinetics revealed two transporters involved in histamine clearance. Extracellular concentration of Na^+/Cl^- did not affect the histamine transport activity by human astrocytes. Drug inhibition assays and gene knockdown assays revealed a major contribution of PMAT and a minor contribution of OCT3 to histamine transport by primary human astrocytes (Fig. 2). This report revealed the molecular mechanism of histamine clearance by astrocytes (Fig. 3) for the first time, although the possible involvement of neurons in histamine clearance cannot be ruled out. We also used microdialysis to examine the importance of polyspecific transporters for histamine clearance around the mouse hypothalamic area in vivo. Imipramine, which can inhibit all three transporters, can significantly increase histamine concentration (unpublished observation), thereby demonstrating that polyspecific transporters are involved in histamine clearance in the brain. Clearly, further research is essential to reveal the contribution of each transporter to histamine removal in vivo. Because studies using OCT2, OCT3, or PMAT knockout mice have already been reported, experiments using these knockout mice will accelerate research on brain histamine clearance. The development of specific inhibitors against OCT2, OCT3, and PMAT is also necessary to evaluate the contribution of each transporter to histamine clearance. Recently, several groups reported that HNMT polymorphism was associated with Parkinson's disease,

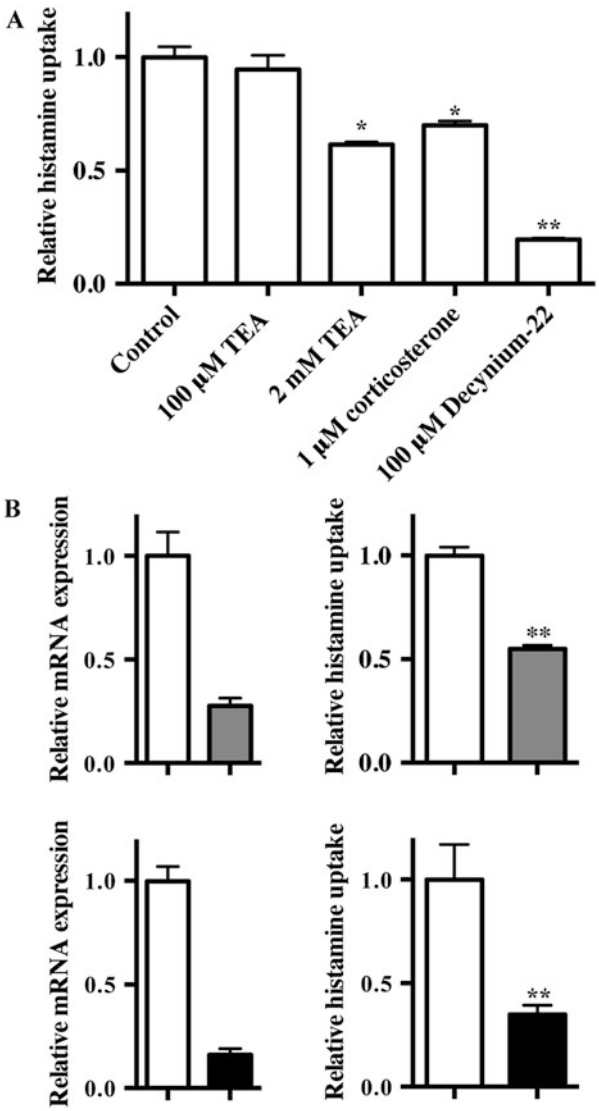

Fig. 2 Histamine transport through OCT3 and PMAT. (**a**) The inhibitory effects of various compounds on histamine transport activity of primary human astrocytes. (**b**) The effect of OCT3 knockdown on OCT3 mRNA expression (*left*) and histamine transport (*right*). (**c**) The effect of PMAT knockdown on PMAT mRNA expression (*left*) and histamine transport (*right*). $*P < 0.05$; $**P < 0.01$ (Yoshikawa et al. 2013)

schizophrenia, and attention-deficit hyperactivity disorder (Stevenson et al. 2010; Yang et al. 2015). These studies demonstrated that lower HNMT activity had protective effects against the diseases, suggesting that higher concentration of histamine due to lower HNMT activity enhances brain functions. It would be valuable to examine whether OCT2, OCT3, or PMAT gene mutations are involved in neuropsychiatric disorders caused by abnormal histaminergic activity. In addition, it would be interesting to investigate whether the expression level of these transporters changes in neurological diseases. Positron emission tomography could

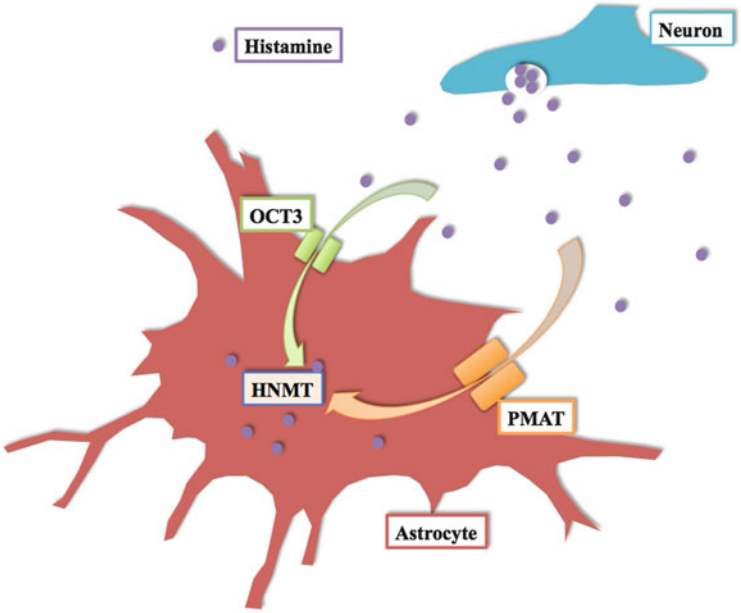

Fig. 3 Molecular mechanism of histamine clearance by human astrocytes. Extracellular histamine is transported through organic cation transporter 3 (OCT3) and plasma membrane monoamine transporter (PMAT). Transported histamine inside the astrocytes is subsequently degraded by histamine N-methyltransferase (HNMT) (Naganuma et al. 2014)

be useful for such studies. Research on histamine clearance could promote better understanding of brain disorders involved in the histaminergic nervous system and might hopefully lead to the development of novel treatments targeting histamine clearance machinery.

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Histidine Decarboxylase Knockout Mice as a Model of the Pathophysiology of Tourette Syndrome and Related Conditions

Christopher Pittenger

Contents

Abstract

While the normal functions of histamine (HA) in the central nervous system have gradually come into focus over the past 30 years, the relationship of abnormalities in neurotransmitter HA to human disease has been slower to emerge. New insight came with the 2010 description of a rare nonsense mutation in the biosynthetic enzyme histidine decarboxylase (*Hdc*) that was associated with Tourette syndrome (TS) and related conditions in a single family pedigree. Subsequent genetic work

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has provided further support for abnormalities of HA signaling in sporadic TS. As a result of this genetic work, *Hdc* knockout mice, which were generated more than 15 years ago, have been reexamined as a model of the pathophysiology of TS and related conditions. Parallel work in these KO mice and in human carriers of the *Hdc* mutation has revealed abnormalities in the basal ganglia system and its modulation by dopamine (DA) and has confirmed the etiologic, face, and predictive validity of the model. The *Hdc*-KO model thus serves as a unique platform to probe the pathophysiology of TS and related conditions, and to generate specific hypotheses for subsequent testing in humans. This chapter summarizes the development and validation of this model and recent and ongoing work using it to further investigate pathophysiological changes that may contribute to these disorders.

Keywords

Animal model • Histamine • *Histidine decarboxylase* • Obsessive–compulsive disorder • Tic disorders • Tourette syndrome

Abbreviations

^{11}C-GSK189254	An H_3 receptor PET tracer
^{11}C-PBR28	A PET tracer that binds to the peripheral benzodiazepine receptor, PBR, a marker of activated microglia
^{11}C-PK11195	A PET tracer that binds to activated microglia
ADHD	Attention deficit-hyperactivity disorder
Akt	Ak-thymoma protein kinase, also known as protein kinase B
ASD	Autism spectrum disorder
AZD5213	An H_3R antagonist
C57Bl/6	C57 Black-6 inbred mouse line
cAMP	Cyclic adenosine monophosphate
CNV	Copy number variation
D_1R	Dopamine D_1 receptor
D_2R	Dopamine D_2 receptor
DA	Dopamine
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein
dMSN	Direct/striatonigral pathway medium spiny neuron
GABA	Gamma-aminobutyric acid
GPe	Globus pallidus, pars externa
GPi	Globus pallidus, pars interna
GSK3beta	Glycogen synthase kinase 3-beta
GWAS	Genome-wide association study

H_1R	Histamine H_1 receptor
H_2R	Histamine H_2 receptor
H_3R	Histamine H_3 receptor
H_4R	Histamine H_4 receptor
HA	Histamine
Hdc	Histidine decarboxylase gene
Hdc-KO	Histidine decarboxylase knockout mouse
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
iMSN	Indirect/striatopallidal pathway medium spiny neuron
JNJ5207852	An H_3R receptor antagonist
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neuron
OCD	Obsessive–compulsive disorder
PANDAS	Pediatric autoimmune neuropsychiatric disorder associated with *Streptococcus*
PET	Positron emission tomography
PHNO	(+)-4-Propyl-9-hydroxynaphthoxazine
PPI	Prepulse inhibition
RAMH	R-aminomethylhistamine, an H4R agonist
SMA	Supplementary motor area
SNc	Substantia nigra, pars compacta
SNr	Substantia nigra, pars reticulata
STN	Subthalamic nucleus
Th1	Type-1 T-helper cell
TS	Tourette syndrome

1 Introduction

Histamine (HA) is a biogenic amine that has long been appreciated to have important roles in the periphery, particularly in the regulation of inflammation (Falus et al. 2004). It was recognized as a neurotransmitter in 1984, when Panula and colleagues identified HA-positive neurons in the posterior hypothalamus (Panula et al. 1984). Since that time, a substantial literature has examined the functions of neurotransmitter HA throughout the brain (Haas et al. 2008; Panula and Nuutinen 2013).

A new window into the role of histamine dysregulation in neuropsychiatric disease was opened by a landmark 2010 genetic study. A combination of linkage analysis and exome sequencing in a family with an exceptionally high incidence of Tourette syndrome (TS), together with a range of comorbid conditions, identified a rare nonsense mutation in the gene *histidine decarboxylase* (*Hdc*) that segregated with the TS phenotype (Ercan-Sencicek et al. 2010). *Hdc* encodes the enzyme that converts the amino

acid histidine into HA and is essential for HA biosynthesis in mammals (Haas et al. 2008). This genetic finding represented the first time that HA dysregulation had been associated with TS.

The TS-associated *Hdc* mutation has a number of characteristics that make it particularly well suited for study in animals, as further elaborated below. *Hdc* knockout mice were generated 15 years ago by Ohtsu and colleagues (Ohtsu et al. 2001) and had been studied in a variety of contexts, but they had not been conceived as a model of TS prior to 2010. Since then, a number of studies have examined these mice, a potential model of the pathophysiology of TS. Studies to date have established the validity of the model at several levels (Castellan Baldan et al. 2014), motivating ongoing work to use these animals as a platform for further investigations of the pathophysiology of TS and related disorders. This work is summarized in this chapter.

2 Clinical Features and Pathophysiology of Tic Disorders

Tics are sudden, rapid, recurrent, nonrhythmic, semi-voluntary movements. Simple tics include such movements as blinking, sniffing, grunting, and turning the head; they are most common in the face but can affect any part of the body. Tics can also be more complex and can incorporate multistep head, arm, or trunk movements and more complex utterances, including complete words or phrases. The spasmodic production of profanity, or coprolalia, is rare but represents a particularly striking form of complex vocal tic. Tics are described as semi-voluntary, because most individuals (especially adults) are aware of a sense of tension or discomfort preceding the tic; this is known as a "premonitory urge." A tic discharges this tension, much as a sneeze discharges a growing discomfort in the back of the nose. Most individuals with tics can suppress them to an extent; however, as with a sneeze, suppressing a tic requires effort and is typically accompanied by increasing discomfort. Tics are lessened by relaxation, sleep, and focused concentration; they are worsened by stress and sleep deprivation (Du et al. 2010; Leckman 2002).

Tics are common, occurring in mild forms in approximately 20% of young people; clinically significant tics occur in about 5%. Tourette syndrome consists of chronic motor and vocal tics, beginning in childhood and persisting for at least a year; it affects ~1% of the population (Robertson et al. 2009; Scahill et al. 2001). Tics and TS are more common in males, with a sex ratio of ~3:1 (Scahill et al. 2001; Scharf et al. 2012). They are also more common in children; approximately 75% of children with a clinically significant tic disorder will improve to the point that they no longer have clinically significant tics by young adulthood (Leckman 2002).

"Pure" TS is uncommon: up to 90% of individuals with a diagnosis of TS carry at least one additional diagnosis, most commonly obsessive–compulsive disorder (OCD) and attention deficit-hyperactivity disorder (ADHD) (Hirschtritt et al. 2015). Tics are also commonly seen in individuals with autism spectrum disorder (ASD) (Canitano and Vivanti 2007). Given this high level of comorbidity, the pathophysiology of tics can be expected to overlap with that of some of these other conditions. A relationship with OCD is particularly clear and has been the subject of considerable study (Pittenger

2017). TS and OCD often run together in families and have some shared genetic risk (Davis et al. 2013; Du et al. 2010). Both are associated with dysregulation of the cortico-basal ganglia circuitry (Leckman et al. 2010; Maia et al. 2008).

Current understanding of the neurobiology of TS is limited. Structural neuroimaging studies have implicated the striatum and afferent cortical areas: the caudate and putamen are slightly but significantly smaller in both children and adults with TS, and afferent sensorimotor cortical areas are thinner (Leckman et al. 2010; Pittenger 2017). Functional neuroimaging suggests phasic abnormalities in activity in this circuitry; tics are associated with increased activity in motor and premotor areas and in the putamen, while effortful tic suppression is associated with activity in more anterior frontal areas and in the caudate. The supplementary area (SMA) is particularly clearly implicated in TS: activity in the SMA uniquely differentiates tics from topographically similar volitional movements (Hampson et al. 2009), and stimulation of the SMA in humans produces both tic-like movements and accompanying urges (Fried et al. 1991).

Several pathophysiological theories of TS, which are by and large not mutually exclusive with one another, have been advanced (Pittenger 2017). One proposal is that TS is associated with elevated dopamine (DA) tone in the striatum. This proposal is based on several observations. First, dopamine D_2R receptor blockers are the most efficacious pharmacotherapy for tics (though their use is limited by their side effects) (Bloch 2008). Second, psychostimulant drugs and DA agonists can trigger stereotypic behaviors in rodents that have been interpreted as tic-like (Canales and Graybiel 2000); supratherapeutic psychostimulant challenge can trigger or transiently worsen tics in patients (Denys et al. 2013; Feinberg and Carroll 1979). Third, some neurochemical imaging studies, though not all, suggest elevated basal and evoked dopamine release in the striatum in patients with tics (Denys et al. 2013; Singer et al. 1992; Wong et al. 2008).

The striatum is the largest nucleus of the basal ganglia and their primary input; in primates, it consists of the caudate and putamen, though these are not discrete structures in rodents. Projections to the striatum and thence to the deeper components of the basal ganglia have classically been described as consisting of two parallel systems, termed the direct and indirect pathway. This scheme is a simplification but is of considerable heuristic value; it appears to be particularly applicable in the dorsal striatum (Fig. 1). Striatal medium spiny neurons (MSNs) of the direct pathway (dMSNs) express D_1R dopamine receptors and have a polysynaptic disinhibitory effect on the thalamic output of the basal ganglia system. MSNs of the indirect pathway (iMSNs) express D_2R dopamine receptors and polysynaptically inhibit the thalamus. Recent data support the idea that these two pathways work in synergy in the process of action selection, with the direct pathway promoting a selected action through disinhibition of relevant thalamocortical feedback, while the indirect pathway inhibits off-target actions through thalamic inhibition (Cui et al. 2013; Hikosaka et al. 2000; Mink 2003). In TS, a modest elevation of tonic DA is likely to primarily affect D_2R receptors on iMSNs, because the D_2R receptor has a much higher affinity for DA than the D_1R receptor. The D_2R receptor reduces firing of iMSNs, and so increased D_2R tone is

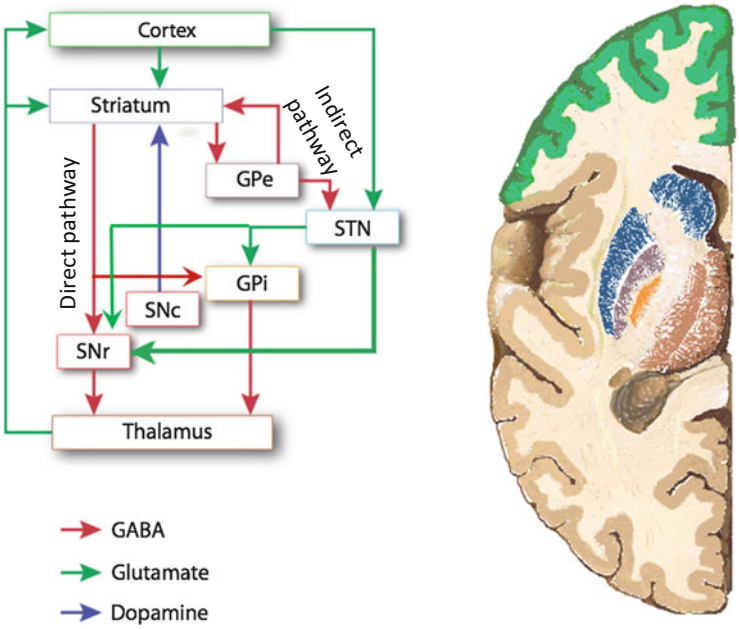

Fig. 1 Major pathways through the cortico-basal ganglia circuitry. Dysregulation of the cortico-basal ganglia circuitry is implicated in TS and tic disorders, as well as in OCD and related conditions (Leckman et al. 2010; Maia et al. 2008; Pittenger 2017). Projections from the cortex and thalamus through the nuclei of the basal ganglia can be conceptualized as traversing two pathways: the direct pathway, which polysynaptically disinhibits thalamic feedback to cortex, and the indirect pathway, which polysynaptically inhibits this feedback. Balance between these two pathways is regulated by dopamine and, perhaps, by histamine. In the *Hdc*-KO model of TS pathophysiology (and, it is proposed, in TS and tic disorders in humans), both DA dysregulation and HA deficiency lead to hyperactivity in the direct pathway and hypoactivity in the indirect pathway; the latter, in particular, may lead to deficient inhibition of off-target action patterns, which may manifest as tics and other repetitive behaviors. See text for further details. Adapted from Pittenger et al. (2011)

predicted to lead to disinhibition of off-target behaviors – which may, it has been proposed, manifest as tics (Albin et al. 1989; Mink 2001, 2003; Pittenger 2017).

A related model is that TS is associated with abnormal inhibition in the striatum. More specifically, localized foci of disinhibition have been proposed to produce domains of autonomous neuronal firing, which manifest as tics (Albin and Mink 2006). This has been directly tested in animals: injection of GABA-A receptor antagonists into the monkey striatum produces tic-like movements of the contralateral limb and face (Bronfeld and Bar-Gad 2013; McCairn et al. 2009). Similar phenomena have been documented in rats and mice (Bronfeld et al. 2013; Pogorelov et al. 2015). Postmortem studies of individuals with severe, refractory tics have documented interneuronal abnormalities in the striatum, providing a potential explanation for deficient inhibition (Kalanithi et al. 2005; Kataoka et al. 2010; Lennington et al. 2016). And targeted disruption of inhibitory interneurons in otherwise normal mice enhances repetitive

movements, providing support for a causal role for disrupted intrastriatal inhibition in the development of tics (Xu et al. 2015a, 2016).

A final perspective on the pathophysiology of TS, OCD, and related conditions, somewhat less well specified than the preceding, focuses on the dysregulation of neuroinflammatory processes. This focus derives from the observation that individuals with TS often exhibit other evidence of dysregulated immune function (Elamin et al. 2013). An extreme example of this is seen in the syndrome of pediatric autoimmune neuropsychiatric disorder associated with *Streptococcus* (PANDAS), in which an autoimmune reaction triggered in a susceptible host by a Streptococcal infection is thought to lead to basal ganglia inflammation (Williams and Swedo 2015). But activation of microglia, the brain's principle inflammatory cells, has also been seen in TS more generally, both in vivo (as measured by PET imaging using a marker of microglial activation (Kumar et al. 2015)) and postmortem (Lennington et al. 2016). Furthermore, individuals with TS, as a group, exhibit abnormalities in a number of peripheral immunological markers (Elamin et al. 2013). Several animal models have demonstrated that experimentally induced microglial abnormalities can produce repetitive behavior, typically elevated grooming (Chen et al. 2010; Zhan et al. 2014). Thus, while the details remain to be established, microglial activation and dysregulated neuroimmune interactions are an increasing focus of interest in the study of TS pathophysiology (Frick and Pittenger 2017).

These various pathophysiological considerations allow us to enumerate a number of testable predictions that can be investigated in any model of TS. In addition to behavioral phenotypes (e.g. repetitive movements), a valid model of TS may be expected to exhibit the modest elevations in tonic striatal DA, tonic or phasic alterations in striatal neuronal activity, abnormalities in striatal inhibition, and possibly abnormalities in neuroinflammatory processes.

3 Animal Models of Tic Disorders

A number of studies over the past 30 years have sought to model tic pathophysiology in animal models (Godar et al. 2014; Pittenger 2014). Analysis of the *Hdc*-knockout model, described in more detail below, has drawn on approaches and principles established in this previous work, which motivates discussion of past models here. It is important to acknowledge at the outset that no animal model of TS (or of OCD, or of any other complex neuropsychiatric condition) should be expected to recapitulate the human syndrome in its entirety (Pittenger 2014; Pittenger et al. 2017).

There are several reasons for this. First, human neuropsychiatric syndromes are themselves complex and heterogeneous categories that may not represent natural kinds and are likely to be recharacterized and recategorized as understanding of pathophysiology advances (Insel and Cuthbert 2015). Second, important aspects of TS are not readily assessed in an animal: for example, repetitive, tic-like behaviors can be observed and quantified, but it is impossible to assess whether they are associated with the premonitory urges that are characteristic of tics. Conversely, it may be unclear what human symptom (if any) a repetitive behavior in an animal best recapitulates: a

repetitive behavior such as elevated grooming (Kalueff et al. 2016) could be homologous to tics, but it could as easily be argued to recapitulate symptoms of autism (Peca et al. 2011), OCD (Greer and Capecchi 2002; Shmelkov et al. 2010; Welch et al. 2007), trichotillomania (Feusner et al. 2009), or some other condition. It is thus perilous to interpret the disease relevance of an animal model or of a particular behavioral phenotype based solely on its resemblance to human symptomatology (that is, on its face validity) (Pittenger et al. 2017).

Finally, while the overall anatomical organization of the cortico-basal ganglia system is preserved between humans and rodents, there are key differences, such as the prominence of the globus pallidus interna (equivalent to the entopeduncular nucleus, which is fairly rudimentary, in rodents), and the fraction of basal ganglia output that projects to thalamus (predominant in humans) versus midbrain and brainstem structures (predominant in rodents). Because of these differences, even a rodent model that captures core pathophysiology perfectly might have behavioral consequences that are not fully isomorphic to tics.

For these reasons, it is better to speak of models that capture aspects of the pathophysiology of a disorder, rather than a disorder in its entirety. Such models are at their strongest when they are based on a clear causal hypothesis – that is, when they have clearly specified construct or etiologic validity (Pittenger et al. 2017). A series of such models have been described in TS and are contributing to increased understanding of the disorder (Godar et al. 2014; Pittenger 2014, 2017). The most informative models can be understood as testing specific hypotheses of the pathophysiology of TS.

As noted above, pharmacological treatments that increased dopamine or dopamine receptor tone, such as psychostimulants, produce repetitive stereotypic behaviors that have some characteristics of tics (Iversen and Creese 1975; Lyon and Robbins 1975). This phenomenon provided early support for the idea that elevated dopamine levels may explain, or at least contribute to, the development of tics. These stereotypic movements after psychostimulant treatment have been observed to correlate with preferential activation of striosomes, neurochemically and synaptically distinct patches of cells within the striatum (Canales and Graybiel 2000). Whether tics correspond to differential activity in striosomes in humans is difficult to test and has not been clearly established, and the validity of amphetamine-induced stereotypies as a model of tics has been questioned (Pittenger 2014).

As noted above, neuroimaging data suggest that corticostriatal circuits are dysregulated and hyperactive in both TS and OCD (Leckman et al. 2010; Maia et al. 2008). Experimental activation of these circuits constitutes a test of the hypothesis that such dysregulation can lead to repetitive, tic-like behaviors. This was first done in a transgenic model described by Burton and colleagues almost 20 years ago (Campbell et al. 1999; Nordstrom and Burton 2002). They expressed a transgene that increases neural activity – the alpha subunit of the cholera toxin – in a subset of D_1R-expressing neurons in the forebrain. This leads to hyperactivity of both cortical and amygdalar projections to the striatum, and corresponding behavioral perseveration, grooming abnormalities, repetitive jumping, and other abnormalities. More recently, a more precise optogenetic approach has been used to perturb cortical projections to the striatum (from the orbitofrontal cortex, in this case); brief daily stimulation of striatal

afferents has been found to result in persistently elevated repetitive behavior (grooming) (Ahmari et al. 2013). In neither of these cases are the repetitive behaviors wholly isomorphic to tics; but the ability of experimentally induced dysregulation of the corticostriatal circuitry to produce repetitive behaviors that support the association of abnormal corticostriatal activity with TS-relevant phenomenology.

Animal model evidence that disrupted local inhibition within the striatum can produce tic-like repetitive behavioral pathology (Bronfeld et al. 2013; McCairn et al. 2009; Pogorelov et al. 2015) or elevated grooming (Xu et al. 2015b, 2016) is reviewed above. These studies confirm the ability of inhibitory deficits within the basal ganglia circuitry to produce TS-relevant effects.

4 Genetics of TS: A Focus on Rare Genes of Large Effect

TS is substantially genetic; recent estimates place heritability at approximately 50% (Davis et al. 2013). However, specific genetic risk factors have been slow to emerge (Fernandez et al. 2017). The one genome-wide association study (GWAS) reported to date identified a few suggestive associations, but none that reached the statistical threshold of genome-wide significance (Scharf et al. 2013).

Common risk alleles of small effect size will no doubt emerge from GWAS analyses as more subjects are studied. However, such mutations are of limited value in the modeling of pathophysiology in animals: recapitulation in an animal of a mutation that increases the risk of developing TS only modestly is likely to have very subtle effects. For a mutation to recapitulate pathophysiology in an animal model, it should ideally have a large effect size, such that carriers are extremely likely to develop disease (i.e., the mutation can be described as a cause of disease, not just a risk factor). Such mutations are invariably rare, and thus difficult or impossible to identify using GWAS methods. Despite their rarity and the attendant challenges of discovering and characterizing them, investigation of such rare mutations of large effect has proven to be of substantial value in other contexts (Geschwind and State 2015).

In TS, several genes have been identified in which rare mutations of large effect are potentially causative (Fernandez et al. 2017). The first to be described, *Slitrk1*, was identified in a patient in which the gene was disrupted by a chromosomal translocation. Subsequent work identified a nonsense mutation and a mutation disrupting a 3′ regulatory site on the mRNA, both of which were associated with TS (Abelson et al. 2005). The functions of *Slitrk1* are not well understood, but it is expressed at high levels in the developing brain (Stillman et al. 2009) and can regulate dendritic outgrowth (Abelson et al. 2005). Despite the genetic evidence that mutations in this gene can cause TS, knockout mice have mood and anxiety phenotypes and have not been reported to exhibit abnormal movements (Katayama et al. 2010). This animal model has yet to shed light on TS pathophysiology.

In contrast, a second rare mutation associated with TS, in the gene *Hdc*, has produced a highly informative animal model. This is the focus of the remainder of this chapter.

5 *Histidine Decarboxylase* Mutations and Other Disruptions of HA Neurotransmission in TS

In 2010, State and colleagues described a two-generation pedigree in which a father and eight children all had chronic tics or TS (Ercan-Sencicek et al. 2010). The mother and her extended family had no history of TS, OCD, or related diagnoses. Linkage analysis in this pedigree identified a single interval, on chromosome 15, that segregated with the TS phenotype. Exome sequencing of this interval identified a single coding-frame mutation: a nonsense mutation, W317X, in *histidine decarboxylase*. This mutation truncates the protein and renders it catalytically inert – in fact, in vitro evidence suggests that the truncated protein functions as a dominant negative, inhibiting the ability of wild-type protein to catalyze the conversion of histidine into histamine (Ercan-Sencicek et al. 2010).

This study focused attention on the potential role of HA dysregulation in the development of TS for the first time (Bloch et al. 2011). However, the *Hdc* W317X mutation is vanishingly rare. Two subsequent genetic studies support the possibility that HA dysregulation contributes to TS more broadly (though still, most likely, in a minority of cases). First, Fernandez and colleagues performed a copy number variation (CNV) analysis in individuals with TS (Fernandez et al. 2012). While *Hdc* itself was not disrupted by any of the detected CNVs, unsupervised pathway analysis of genes affected by CNVs in TS implicated disruption of HA-mediated signaling. Second, Karagiannidis and colleagues examined markers of common variants at the *Hdc* locus in several hundred individuals with TS, and matched controls, and found overtransmission of a particular haplotype in patients; this suggests a contribution of common variants at this locus to disease risk (Karagiannidis et al. 2013). HA dysregulation is almost certainly still a rare cause of TS, but these findings suggest that it is not unique to the originally described *Hdc*-W317X family (Ercan-Sencicek et al. 2010).

To be harnessed for studying pathophysiology in an animal model, a disease-associated mutation should ideally have several characteristics; the *Hdc*-W317X mutation has all of them and is thus particularly well suited for reverse translational analysis. First, as noted above, a disease-associated mutation is most likely to yield insights into pathophysiology if it has a large effect on disease risk. In the case of the *Hdc*-W317X mutation, every carrier who has been characterized to date (all in the originally described family) has TS or chronic tics, suggesting a large effect. Second, the mutation ideally has a known, quantifiable effect on a gene of known function. This is true in the case of *Hdc*-W317X: the function of the encoded enzyme is known (it is critical for the biosynthesis of HA), and the effect of the mutation is well established and quantifiable (it completely abrogates HA biosynthesis). Finally, a disease-associated mutation is more convincing if it implicates systems with a plausible link to established pathophysiology. While a link between HA neurotransmission and TS was not contemplated until a few years ago, the link is a priori plausible: as reviewed elsewhere (Haas et al. 2008; Panula and Nuutinen 2013), including in other chapters in this volume, neurotransmitter HA modulates DA (Castellan Baldan et al. 2014; Schlicker et al. 1994) and basal ganglia function (Bolam and Ellender 2015), both of which are implicated in TS.

6 The *Histidine Decarboxylase* Knockout Mouse as a Model of TS Pathophysiology: Initial Validation

These considerations have motivated examination of mice in which the *Hdc* gene is mutated as a potential model of the pathophysiology of TS (Table 1). Initial work has not recapitulated the W317X mutation but rather has examined mice in which the *Hdc* gene is inactivated using conventional knockout technology; these mice were first described 15 years ago (Ohtsu et al. 2001). *Hdc* full knockout mice are unable to synthesize HA; while some studies have suggested low persistent levels of HA (Ohtsu et al. 2001), in recent studies, HA levels in the brain are so low as to be undetectable (Castellan Baldan et al. 2014). Heterozygotes have intermediate levels of HA in brain (Castellan Baldan et al. 2014), which is important: while it has not been possible to directly assay HA levels in brain in human carriers of the *Hdc* W317X mutation, they are presumably reduced, but not zero. Therefore, while *Hdc* full knockout mice are useful probes of pathophysiology, heterozygotes may be closer to the human disease state; they have been included in some, but not all, of the analyses discussed here. This consideration also reduces the importance of any potential residual HA in the brains of KO mice: the presence of low levels of HA, below what the level of detection, does not undermine the utility of these animals as a tool to probe processes of potential relevance to TS pathophysiology.

Table 1 TS-relevant measures in humans and mice carrying mutations in *Hdc*

Characteristic	Patients w/*Hdc* W317X mutation	*Hdc* +/− & −/− mice	References
Histamine biosynthesis	Reduced (in vitro)	Reduced in tissue and striatal microdialysate	Ercan-Sencicek et al. 2010; Castellan Baldan et al. 2014; Ohtsu et al. 2001
Tics/ stereotypy	Motor, phonic tics	Potentiated stereotypy after threshold-dose amphetamine and after stress	Ercan-Sencicek et al. 2010; Castellan Baldan et al. 2014; Xu et al. 2015b
Prepulse inhibition	Reduced	Reduced	Castellan Baldan et al. 2014
Striatal dopamine	Not directly measured	Increased in active-phase microdialysate	Castellan Baldan et al. 2014; Rapanelli et al. 2014
Striatal dopamine signaling	Not directly measured	Increased striatal *Fos* expression at baseline and after amphetamine	Castellan Baldan et al. 2014; Rapanelli et al. 2014
Substantia nigra D2/D3 binding	Increased by in vivo PHNO PET imaging	Increased by in vitro raclopride binding	Castellan Baldan et al. 2014
Dorsal striatal D2/D3 binding	No evident change, by in vivo PHNO PET imaging	Modest decrease, by in vitro raclopride binding	Castellan Baldan et al. 2014

As noted, *Hdc* knockout mice were generated years ago, and they have been characterized in a range of behavioral and neurochemical experiments, by a number of different authors (Schneider et al. 2014); they have also been extensively characterized in assays of inflammatory and immune processes (Ohtsu 2010). Some findings may be interpreted, in retrospect, as being of relevance to the pathophysiology of TS. For example, *Hdc*-KO mice have been reported to have increased DA turnover in the striatum, suggestive of altered dopaminergic modulation (Dere et al. 2003). Other studies have examined anxiety-like, depression-like, learning, and other phenotypes, with variable results (Acevedo et al. 2006a, b; Dere et al. 2004; Schneider et al. 2014).

At baseline, no tic-like movements, elevated grooming, or any other repetitive behaviors of potential relevance to TS were evident in *Hdc*-KO mice. Exploratory behavior in an open field, rearing, anxiety-like behavior (Castellan Baldan et al. 2014), and fear conditioning (Xu et al. 2015b) were normal. This normal baseline behavior is at odds with some previous reports (Acevedo et al. 2006a; Dere et al. 2004). One possible explanation for this discrepancy is that different investigators have examined these mice on different genetic backgrounds (Schneider et al. 2014). These studies have been performed in males, extensively backcrossed (>N9) onto C57Bl/6. Regardless, normal baseline exploratory behaviors in these animals simplify interpretation of other behavioral phenotypes in these experiments.

Tics in TS fluctuate dramatically (Leckman 2002); they are potentiated by such factors as acute stress (Buse et al. 2014; Conelea and Woods 2008), sleep deprivation, and supratherapeutic doses of psychostimulants (Denys et al. 2013; Feinberg and Carroll 1979). To further investigate tic-like phenomenology in the *Hdc*-KO mice, therefore, mice were acutely challenged with a high dose of the psychostimulant D-amphetamine. At a dose that produces locomotor activation but few stereotypies in a wild-type mouse (on this genetic background), stereotypies were markedly enhanced in the KO animals. At a slightly higher dose, many of the KO animals became completely immobile; heterozygotes had elevated stereotypies (Castellan Baldan et al. 2014). Pretreatment with the D_2R antagonist haloperidol, which is an efficacious treatment for tics (Bloch 2008), mitigated these stereotypies, endowing the model with a degree of predictive validity (Castellan Baldan et al. 2014) (Fig. 2a). A similar interactive effect was seen after acute stress, induced by cued fear conditioning: KO animals showed elevated repetitive behavior (grooming, in this case) after the induction of stress, but not at baseline (Xu et al. 2015b) (Fig. 2b).

The face validity of these two repetitive behavioral phenotypes is open to question; certainly neither the repetitive stereotypic sniffing seen after amphetamine challenge nor the elevated grooming seen after stress is as clearly isomorphic to tics as the unilateral, spasmodic, nonrhythmic movements seen after focal striatal inhibition in other models (Bronfeld and Bar-Gad 2013; Bronfeld et al. 2013; McCairn et al. 2009; Pogorelov et al. 2015). However, face validity is a fickle guide in the interpretation of animal models of tic pathophysiology (Pittenger 2014); indeed, as argued above, both the complexity of neuropsychiatric phenotypes and the differences between human and rodent neuroanatomy suggest that even optimal recapitulation of tic pathophysiology in a mouse might produce behavioral effects that do not look identical to human

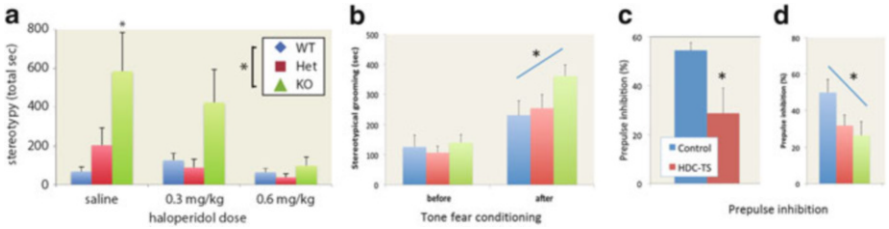

Fig. 2 Stereotypies in *Hdc* KO mice. (**a**) Stereotypies after D-amphetamine (8.5 mg/kg) were potentiated in *Hdc* KO and Het mice; pretreatment with haloperidol mitigated this effect. From Castellan Baldan et al. (2014), with permission. (**b**) Stress, induced by tone fear conditioning, similarly increased stereotypical grooming. Adapted from Xu et al. (2015b). (**c**) Prepulse inhibition (PPI), a measure of sensorimotor gating, was reduced in human carriers of the *Hdc* W317X mutation. (**d**) PPI is similarly reduced in *Hdc* heterozygotes and knockouts. Data are shown for a 6 dB prepulse; similar effects were seen with larger prepulses. (**c**, **d**) from Castellan Baldan et al. (2014), with permission

tics. Therefore, in these experiments, and in other TS models (Xu et al. 2015b, 2016), a range of repetitive behaviors are accepted as tentatively confirmatory of relevance to TS. The claim to relevance to tics derives not from the specific topography of the behavior, but rather from the recapitulation of underlying pathophysiological processes – in this case, disruption of the *Hdc* gene. Put another way: an elevated grooming phenotype in isolation is difficult to interpret with respect to any particular neuropsychiatric diagnosis (Kalueff et al. 2016) and is unavoidably ambiguous; but an elevated grooming phenotype in conjunction with a clear recapitulation of a hypothesized causal factor, like *Hdc* gene disruption (Xu et al. 2015b), may be interpreted, at least provisionally, as confirmatory of the underlying causal hypothesis.

While tics are central to the diagnosis of TS, patients with tics typically have a range of other abnormalities, some of which are described above. Some, like the presence of premonitory urges before tics and the ability to effortfully suppress them, are difficult to assess in an animal model; but others can be assayed across species. A deficit in sensorimotor gating, indexed by prepulse inhibition (PPI), is in the latter category. Individuals with TS have deficient PPI (Castellanos et al. 1996; Kohl et al. 2013; Swerdlow et al. 2001), as do individuals with OCD (Ahmari et al. 2012; Hoenig et al. 2005; Kohl et al. 2013). PPI was tested both in human carriers of the *Hdc*-W317X mutation and in *Hdc*-KO mice. PPI of the acoustic startle reflex was impaired in both, compared to normal controls. In the mice, baseline startle was increased by *Hdc* knockout, but the deficit in PPI persisted after controlling for this effect. Importantly, heterozygotes – which, as noted above, may better recapitulate partial HA deficiency in the patients than do the KOs – showed an intermediate PPI deficit (Castellan Baldan et al. 2014). These PPI findings provide an additional behavioral parallel between TS patients and the *Hdc*-KO model (Fig. 2c).

7 Pathophysiological Mechanisms in the *Hdc*-KO Model: Dopamine and Dopamine Receptors

With this validation in hand, candidate pathophysiological processes in the *Hdc*-KO model were investigated. The initial focus was on dopamine modulation of the striatum; as reviewed above, convergent evidence suggests a modest elevation in tonic striatal DA in patients with tics (Pittenger 2017). Similar effects were predicted in the model.

Direct measurement of tonic extrasynaptic DA levels is possible using in vivo microdialysis. In the knockout animals, baseline striatal DA was elevated (Rapanelli et al. 2014). This baseline elevation was accentuated in the animals' dark phase, when HA is normally elevated in mice (which are nocturnal); HA is of course absent in the KO animals, and thus this enhanced DA elevation in the dark cycle is consistent with negative regulation of DA by HA (Castellan Baldan et al. 2014). To directly test this, the effects of infusion of HA on DA levels were measured in vivo using microdialysis, in wild-type mice. As predicted, intacerebroventricular HA infusion reduced striatal DA levels (Castellan Baldan et al. 2014). This elevation in tonic extrasynaptic DA, which accords with current thinking about TS, provides further confirmation that the *Hdc*-KO model is recapitulating key aspects of pathophysiology.

What is the mechanism of this reduction in striatal DA levels by HA, and of the elevation in DA seen in the KO animals? Histamine binds to four G-protein-coupled receptors, H_1R-H_4R; H_1R-H_3R are expressed on neurons in the central nervous system, while H_4R appears not to be (Haas et al. 2008; Schneider and Seifert 2016). The initial focus was on H_3R. This receptor couples to G_{alpha}-i and has classically been considered to function primarily as a presynaptic inhibitor of transmitter release, both of histamine itself and of other transmitters (Haas et al. 2008). Ex vivo, it has been reported to inhibit DA release (Schlicker et al. 1994). Thus, loss of H_3R tone on DA terminals in KO animals might lead to disinhibited DA release, and HA actions on H_3R receptors on DA terminals might explain the reduced DA seen in vivo after HA infusion (Castellan Baldan et al. 2014).

However, recent data argue against this mechanism. The H_3R agonist immepip has not been found to affect intrastriatal DA levels in wild-type mice (Alfaro-Rodriguez et al. 2013). Similarly, systemic administration of the specific agonist R-aminomethylhistamine (RAMH), at doses that produce behavioral effects (see below; Rapanelli et al. 2017), does not produce the predicted reduction in striatal DA – in fact, in KO mice it produces a small but significant elevation in DA after RAMH challenge (Rapanelli et al. 2016, 2017). The ability of both endogenous and exogenous HA to reduce striatal DA levels (Castellan Baldan et al. 2014) can be concluded to depend on different receptors.

H_1R is a candidate. H_1R antagonists have been found to acutely increase intrastriatal DA (Dringenberg et al. 1998) and to produce rewarding effects in some behavioral paradigms (Halpert et al. 2002; Zimmermann et al. 1999), although the dependence of such effects on binding to H_1R has been questioned (Oleson et al. 2012; Suzuki et al. 1999). The detailed mechanisms of HA regulation of striatal DA remain an important open question.

Elevated striatal DA is expected to produce a number of secondary effects. First, DA can activate of D_1R-expressing dMSNs. Expression of the immediate early genes *c-fos* was modestly elevated at baseline in the striatum in *Hdc*-KO mice (Castellan Baldan et al. 2014), consistent with such an effect – and perhaps paralleling the dysregulation of the corticostriatal circuitry seen in patients with TS (Leckman et al. 2010). *C-fos* is elevated following amphetamine challenge, as one would expect; interestingly, *c-fos* expression is particularly high in striosomes after amphetamine challenge in the knockout, relative to wild-type controls (Castellan Baldan et al. 2014). This parallels the specific role for striosomal MSN activity in stereotypy/tic generation suggested by *fos*-mapping investigations in wild-type mice (Canales and Graybiel 2000).

Elevated striatal DA also has specific effects on molecular signaling within MSNs of both the direct and the indirect pathway (Girault 2012). Selected signaling pathways were examined in *Hdc*-KO mice (Rapanelli et al. 2014). Signaling through the MAPK pathway was elevated in KO mice, consistent with a DA effect in D_1R-expressing MSNs. The kinases Akt and Gsk3beta were relatively dephosphorylated, consistent with a DA effect in D_2R-expresing MSNs. Both effects were further amplified by amphetamine treatment (Rapanelli et al. 2014). These results should be interpreted as preliminary; in particular, these initial studies did not differentiate between MSNs of the direct and indirect pathways. Work to better elucidate specific signaling alterations in these two pathways is ongoing. Additionally, the same pathways can be regulated by postsynaptic H_3R receptors (Rapanelli et al. 2016); this complication is further addressed below.

A third effect of tonic elevation of striatal DA is the development of compensatory changes in DA receptor expression. In particular, treatment with both DA agonists and psychostimulants leads to decreased expression of D_2R receptor in the dorsal striatum and elevated expression of the D_3R receptor in the substantia nigra (Fauchey et al. 2000; Stanwood et al. 2000; Volkow et al. 2009). D_2R and D_3R receptors were examined in *Hdc*-KO mice using in vitro binding with the agonist raclopride. D_2R/D_3R receptor binding was downregulated in dorsal striatum, though the effect was subtle. More dramatic was the upregulation of D_2R/D_3R binding in the substantia nigra in KO mice (Castellan Baldan et al. 2014). These alterations are consistent with the predicted effects of chronic DA excess.

Importantly, while striatal DA levels cannot be directly assessed in humans, DA receptors can be. D_2R/D_3R receptors were examined in TS patients carrying the *Hdc* W317X mutation using positron emission tomography (PET) imaging with the agonist tracer PHNO. This investigation was limited to adult patients; after controlling for imaging quality, three adult carriers of the *Hdc* W317X mutation and nine matched healthy controls were included in the analysis. In this limited sample, there was no detectable alteration in striatal D_2R/D_3R binding. In the substantia nigra, in contrast, there was a striking upregulation of D_2R/D_3R receptor binding (Castellan Baldan et al. 2014). A similar pattern of increased PHNO binding in the nigra has been seen in human cocaine abusers, supporting the idea that it is a consequence of chronic DA receptor hyperstimulation (Matuskey et al. 2015; Payer et al. 2014). PHNO binding in the substantia nigra is thought to primarily reflect D_3R receptor density (Rabiner et al.

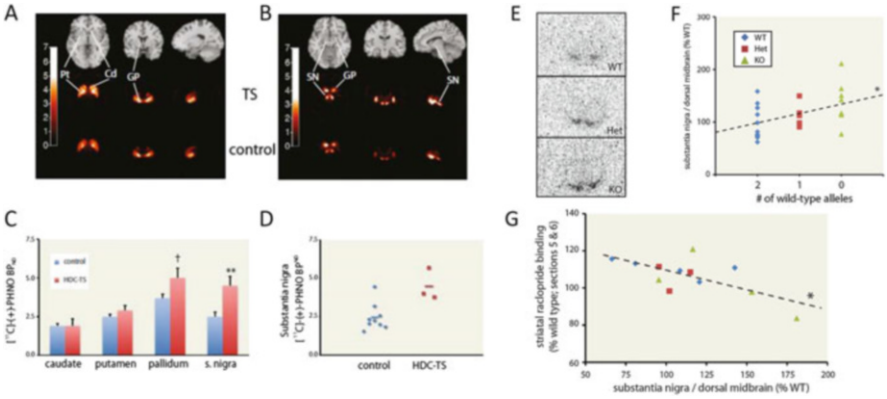

Fig. 3 D2/D3 receptors in humans and mice with a mutated *Hdc* gene. (**a–d**) D2/D3R receptor availability in TS patients carrying the *Hdc*-W317X mutation, relative to matched controls, measured using in vivo ^{11}C-PHNO PET imaging. (**a**, **b**) composite radioligand binding images from patients (*middle row*) and controls (*bottom row*). (**c**) Binding in subnuclei of the basal ganglia. (**d**) Individual subject binding in the substantia nigra in patients and controls; group means are show by a *horizontal line*. (**e–g**) D2/D3R receptor binding in mice measured ex vivo using ^{3}H-raclopride binding. (**e**) Raclopride binding in the substantia nigra. (**f**) Increased binding was seen in *Hdc* het and KO mice; individual data are shown. (**g**) Reduced raclopride binding was seen in dorsal striatum; this correlated negatively, on an animal-by-animal basis, with the increased binding in the nigra. From Castellan Baldan et al. (2014), with permission

2009; Tziortzi et al. 2011), although it cannot be concluded with complete certainty that the observed increase in PHNO binding is due solely to increased D_3R expression. Regardless, the parallel increase in D_2R/D_3R binding in these patients and in *Hdc*-KO mice adds an additional validation of the mice as an informative model of pathophysiology (Fig. 3).

8 Pathophysiological Mechanisms in the *Hdc*-KO Model: Histamine Receptors

HA receptors have been previously examined in *Hdc*-KO mice; for example, H_3R receptors have been reported to be downregulated in hippocampus and upregulated in hypothalamus in these animals (Chepkova et al. 2012). All four HA receptors were examined in the basal ganglia, using both radioligand binding and in situ quantification of mRNA expression (Frick et al. 2016; Rapanelli et al. 2017). H_2R receptors are decreased in the striatum in *Hdc*-KO mice, at the level of ligand binding, but not of mRNA expression; this suggests posttranslational regulation of receptor level, alteration in affinity rather than expression, or decreased expression on afferents (with the corresponding mRNA alterations elsewhere in the brain). H_4R receptors are also decreased, at the level of both mRNA and ligand binding; this alteration is further addressed below. H_1R receptor is unchanged (Frick et al. 2016; Rapanelli et al. 2017).

H_3R receptors in the striatum are increased in KO mice (Rapanelli et al. 2017). H_3R receptors have high constitutive activity, at least in histaminergic neurons themselves (Morisset et al. 2000). This raises the intriguing possibility that elevated H_3R expression may influence striatal function in KO mice even in the absence of its ligand, HA. Systemic administration of the H_3R agonist RAMH produced stereotypies in KO mice. The same effect is produced by the chemically dissimilar agonist immepip; it is blocked by the H_3R antagonist JNJ5207852, further confirming the specificity of the effect (Rapanelli et al. 2017). These observations provide further support for the idea that H_3R activity contributes to tic-like phenomenology in these mice.

As noted above, H_3R has classically been considered a presynaptic receptor negatively regulating transmitter release (Haas et al. 2008; Schlicker et al. 1994); and indeed there is evidence for such a role on glutamatergic afferents to the striatum (Ellender et al. 2011). However, it is increasingly evident that postsynaptic H_3R receptors play a prominent and complex role in the striatum (Bolam and Ellender 2015; Panula and Nuutinen 2013). Postsynaptic H_3R receptors interact physically and functionally with both D_1R and D_2R receptors, and their signaling properties are markedly different in different MSN types (Ferrada et al. 2008, 2009; Moreno et al. 2011).

These observations were confirmed and extended in MSNs in vivo, in wild-type mice (Rapanelli et al. 2016). After acute challenge with the H_3R agonist RAMH, the MAPK signaling pathway is rapidly and transiently activated in D_1R-expressing dMSNs, but not in D_2R-expressing MSNs of the iMSNs. cAMP-dependent modulation of the key regulatory molecular DARPP-32 is not affected by RAMH in either cell type; this is surprising in light of the traditional concept of H_3R as a G_{alpha}-i-coupled receptor, which would be expected to reduce cAMP. Regulation of the Akt–GSK signaling pathway is particularly interesting. DA acting on D_2R receptors in iMSNs inhibits Akt, thus dephosphorylating and thereby activating GSK (Beaulieu et al. 2005). H_3R receptor activation recapitulates this effect. In dMSNs, on the other hand, DA has no effect on Akt–GSK signaling, but H_3R activation activates Akt, thereby phosphorylating and inhibiting GSK (Rapanelli et al. 2016). This differential regulation of Akt/GSK signaling in such similar cell types by H_3R may be unique; its importance is a topic of active investigation.

These abnormalities in signaling in vivo after RAMH challenge in wild-type mice are similar to the basal abnormalities seen in knockout animals (Rapanelli et al. 2014). These signaling abnormalities may relate to elevated tonic DA, as discussed above. However, since H_3R receptors are upregulated in *Hdc*-KO mice, these changes may also result from constitutive effects of H_3R (presuming that postsynaptic striatal H_3R receptors have the same high constitutive activity that has been reported in other contexts; Morisset et al. 2000). These possibilities are not mutually exclusive; DA elevation and H_3R upregulation may have additive or interactive effects, the details of which have yet to be worked out.

9 Pathophysiological Mechanisms in the *Hdc*-KO Model: Modulation of Microglia

As noted above, convergent evidence suggests an immune or neuroinflammatory contribution to TS, at least in some cases (Elamin et al. 2013; Frick and Pittenger 2017; Kumar et al. 2015; Lennington et al. 2016; Williams and Swedo 2015). HA is a regulator of allergic and inflammatory processes, and dysregulation of peripheral inflammatory processes has been extensively investigated in the *Hdc*-KO mice (Ohtsu 2010). This motivated us to investigate the effects of HA on microglia, the primary inflammatory cells in the brain. Previous in vitro investigations of HA regulation of acutely isolated or cultured microglia have produced conflicting results.

These questions were further examined in vivo, in wild-type and *Hdc*-KO mice (Frick et al. 2016). HA infusion into the brain in vivo leads to an increased density of and marked morphological changes in microglia, particularly in the striatum and hypothalamus (Frick et al. 2016). This appears to be mediated by the H_4R receptor (Frick et al. 2016), which is thought to be expressed on microglia but not on neurons (Schneider and Seifert 2016). Conversely, in *Hdc*-KO mice, microglia are normal in number but reduced in their ramifications, and the H_4R receptor is downregulated (Frick et al. 2016), suggesting that HA regulation is important under physiological conditions. (Of note, these studies used a relatively crude measure of microglial process density, the optical density of Iba1 immunostaining; while this measure efficiently reveals differences between groups and between conditions, its relationship to microglial functional "activation" is unclear.)

This latter observation was initially puzzling, as it contrasts with what has been reported in patients with TS: increased activation of microglia, and increased expression of microglial markers (Frick and Pittenger 2017; Kumar et al. 2015; Lennington et al. 2016). A resolution to this conundrum may be seen in the recent distinction between neuroprotective and inflammatory microglia (Olah et al. 2011). Some in vitro studies (Ferreira et al. 2012; Iida et al. 2015), though not all, suggest that HA-stimulated microglia may have a neuroprotective phenotype, and that HA may antagonize the classical inflammatory effects of stimuli such as lipopolysaccharide (LPS). In vivo, *Hdc*-KO mice to have a reduction in the fraction of microglia expressing the neurotrophin IGF-1, which is thought to be a marker of such neuroprotective microglia (Frick et al. 2016). This suggests an intriguing hypothesis, which may have pathophysiological significance: that absence of HA in *Hdc*-KO mice may lead to a deficit in neuroprotective microglia and, perhaps, to a consequent dysregulation of neuroinflammatory responses (Frick and Pittenger 2017).

This hypothesis was tested by administering LPS to *Hdc*-KO mice. As predicted, *Hdc*-KOs showed an overexuberant microglial response to LPS challenge, apparent both in microglial morphology and in the production of the Th1 interleukin IL-1. As a consequence, microglial ramifications, which were reduced at baseline in KOs relative to WT controls, were increased after LPS (Frick et al. 2016). This observation provides a potential explanation for the discrepancy between the apparently quiescent microglia seen at baseline in the KO model and the activated microglia observed in vivo and postmortem in TS (Kumar et al. 2015; Lennington et al. 2016).

With respect to microglial activation, *Hdc* deficiency (and analogous causal factors) may represent a vulnerability factor but may not fully recapitulate the disease state; in patients, who (unlike vivarium-raised mice) are subject to a lifetime of immune challenges, this may interact with viral infections and other pro-inflammatory stimuli to unmask neuroinflammatory dysregulation.

This "two-hit" model of microglial dysregulation (Frick et al. 2016; Frick and Pittenger 2017) suggests that face-valid behavioral phenotypes may be more evident after inflammatory challenge – perhaps even that behavioral stereotypy, elevated grooming, or other TS-relevant behavioral pathology might emerge spontaneously in LPS-challenged mice. Tests of this hypothesis to date have been equivocal (unpublished data); this work is ongoing.

10 Interpreting the *Hdc*-KO Model: What Human Condition(s) Are Being Recapitulated?

Several different lines of analysis in this model system are summarized above, one or more of which may prove to reflect events that are occurring in patients. Analysis in any such model system is best seen as recapitulating aspects of pathophysiology, and not as capturing TS, or any other particularly disease entity, in its entirety. With this caveat, it may be asked which patients are most likely to manifest similar mechanisms.

Patients carrying the *Hdc* W317X mutation all have TS (or at least chronic tics; in one of the two papers describing these patients, one subject was diagnosed with chronic tics rather than the full syndrome of TS) (Castellan Baldan et al. 2014; Ercan-Sencicek et al. 2010). But, as is typical for TS, most have comorbidities: four OCD (two full syndromes and two subclinical); three depression; one ASD; three social phobia; one trichotillomania, and one ADHD. Thus, the mutation is not associated with tics specifically, but rather with a more complex and somewhat heterogeneous clinical syndrome.

Certain abnormalities seen in the *Hdc*-KO model that can be assayed across species are seen in carriers of the W317X mutation: in particular, PPI deficits and elevated D_2R/D_3R binding in the substantia nigra (Castellan Baldan et al. 2014). But it remains possible that findings in the *Hdc*-KO model will generalize only to patients with this or similar rare mutations affecting brain histamine. Such limited generalizability would obviously reduce the clinical impact of work in the model. Alternatively, findings from the *Hdc*-KO system may generalize to some or all patients with tics, or more broadly, to OCD, ADHD, or other related conditions. This is, ultimately, an empirical question, which has yet to be resolved. The answer may differ for distinct findings in the model system: for example, some candidate pathophysiological mechanisms identified in the model may be seen only in patients with tics; others may be seen in a broader range of clinical groups; and still others may have no relevance to human disease at all.

11 Closing the Loop: Testing Hypotheses from the Hdc-KO Model in Patients

The foregoing discussion reemphasizes that, from a translational perspective, such a model system is best considered a generator of pathophysiological hypotheses for testing in humans, and not as a veridical recapitulation of a particular disease or syndrome in its entirety. Ultimately, the translational value of such a pathophysiological model lies in its ability to generate hypotheses about human disease that would not otherwise have been considered, with the ultimate goal of advancing disease diagnosis, treatment, or prevention. With this in mind, it is important to identify abnormalities in the *Hdc*-KO mouse system (and especially in *Hdc* heterozygotes) that are testable in humans.

One of these is shown in Fig. 2: elevated D_2R/D_3R availability can be measured in vivo in humans using ^{11}C-PHNO PET imaging, and patients carrying the W317X mutation have an abnormality that parallels that seen in the KO mice (Castellan Baldan et al. 2014). It remains to be seen whether a similar abnormality is seen in patients with TS or tics more generally. The fact that similarly increased nigral D_2R/D_3R binding is seen in cocaine users (Matuskey et al. 2015; Payer et al. 2014) suggests that this may be a marker of chronic DA receptor hyperstimulation (Fauchey et al. 2000; Stanwood et al. 2000), and not of tic pathophysiology specifically: that is, elevated PHNO binding may be informative with regard to mechanism, but of limited clinical specificity.

Two other of the findings described above are potentially amenable to in vivo testing using PET imaging in humans. First, H_3R upregulation in the striatum, which is seen in the *Hdc*-KO mice and may be of importance in the generation of their repetitive behavioral pathology (Rapanelli et al. 2017), can be assayed in humans using the PET ligand ^{11}C-GSK189254 (Gallezot et al. 2016). This has not yet been done in patients with TS, OCD, or related conditions. Interestingly, the H_3R gene is nominally upregulated in postmortem tissue from adults with TS, though not to a degree that emerges with statistical significance from the limited studies that have been reported to date (Lennington et al. 2016, supplemental data).

Another finding from the *Hdc*-KO model that can be tested in patients, in principle, is the activation of microglia seen after inflammatory challenge (Frick et al. 2016); this can be tested using in vivo PET imaging with the radioligands ^{11}C-PBR28 (Sandiego et al. 2015) or ^{11}C-PK11195 (Kumar et al. 2015), which bind to markers of microglial activation. Indeed, imaging in children with TS using ^{11}C-PK11195 has revealed elevated binding in the basal ganglia, relative to healthy adult controls (Kumar et al. 2015). Further studies will be needed to establish the generality of this abnormality.

Clinically, one ultimate translational goal of such a model is the ability to identify novel therapeutic targets. One candidate target emerges from the studies described above: the histamine H_3R receptor (Rapanelli and Pittenger 2016). It is not yet clear how the H_3R receptor might best be modulated to mitigate tic-like stereotypy; but the ability of an H_3R agonist to elicit repetitive behavioral pathology in the *Hdc*-KO system (Rapanelli et al. 2017) suggests that H_3R antagonism might be therapeutic. Indeed, one recent clinical study has investigated the efficacy of an H_3R antagonist/inverse

agonist, AZD5213. Surprisingly, in this small clinical trial, H_3R antagonism produced a small but statistically significant *worsening* of tics (www.clinicaltrials.gov: NCT01904773). This supports the relevance of the H_3R receptor for the pathophysiology of TS beyond the original W317X family, but it indicates that further work is needed to clarify how this receptor might best be targeted to produce therapeutic benefit.

12 Conclusion

The association of HA dysregulation with TS and related conditions emerged only recently. Mechanistic work focusing on disease-relevant abnormalities in the *Hdc*-KO model has advanced significantly but remains in its early stages. The initial validation of the model has been summarized (Castellan Baldan et al. 2014), and recent advances in three areas have been described: dopamine dysregulation and abnormalities in DA receptors; abnormalities in HA receptors, especially in H_3R; and dysregulation of microglia and neuroinflammatory processes. Many questions remain in each of these domains.

Most importantly, the translation of these observations back to clinical subjects is incomplete. In the coming years, it is to be hoped that pathophysiological hypotheses generated in the *Hdc*-KO system, and related models, will be testable in patients, and will lead to new insights into the fundamental nature of tic disorders and to new strategies for mitigation or prevention.

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Histamine Food Poisoning

Maria Schirone, Pierina Visciano, Rosanna Tofalo, and Giovanna Suzzi

Contents

Abstract

The consumption of food containing high amounts of histamine and other biogenic amines can cause food poisoning with different symptoms linked to the individual sensitivity and the detoxification activity. Histamine is the only biogenic amine with regulatory limits set by the European Commission in fish and fishery products, because it can lead to a fatal outcome. However, also fermented foods can be involved in outbreaks and sporadic cases of intoxication. The factors affecting the presence of histamine in food are variable and product specific including the availability of the precursor amino acid, the presence of microorganisms producing decarboxylases, and the conditions allowing their growth and enzyme production. Generally, the good quality of raw material and hygienic practices during food processing as well as the use of histidine decarboxylase-negative starter cultures can minimize the occurrence of histamine. Further studies are necessary to estimate the human exposure and the

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relationship between the total amount of the biogenic amines ingested with food and health effects.

Keywords
Cheese • Decarboxylase-positive bacteria • Fish • Histamine • Sausages • Wine

1 Introduction

Histamine and other biogenic amines (BAs) can be present in foodstuffs both of animal and vegetable origin providing a good indication of their quality. Their formation is linked to the availability of the corresponding free amino acid, the presence of decarboxylase-positive microorganisms, and favorable conditions for their growth and decarboxylation activity. Free amino acids can occur as such in food or can be derived from proteolysis during storage of raw material or manufacturing processes as maturation and ripening (Schirone et al. 2014).

Many different microorganisms are potential BA producers, both Gram-positive and Gram-negative bacteria and yeasts. Besides *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* identified in fishery products, other bacterial species are known to possess BA decarboxylase such as lactic acid bacteria (LAB) including the genera *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus* but also *Staphylococcus* and *Kocuria* in dairy and meat products and *Oenococcus oeni* and *Lactobacillus hilgardii* in wine. Within microbial groups the capacity to produce BAs is however a strain-specific characteristic, more widely distributed among certain genera or species, suggesting that horizontal gene transfer may account for their dissemination between strains. In mammals and other eukaryotic organisms, as well as in Gram-negative bacteria, histidine decarboxylase (HDC) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme (Moya-García et al. 2009), whereas in Gram-positive bacteria, the reaction catalyst is a pyruvoyl-containing enzyme (Schwelberger et al. 2013). Conditions that favor histidine decarboxylation are the presence of the specific substrate or high concentrations of free amino acids and hostile environmental conditions that require the maintenance of pH homeostasis (Rossi et al. 2011). Kinetic studies on pure enzyme confirmed a pH optimum at 4.8, with a light inhibition at high histidine concentration, while at pH 7.6 HDC showed a sigmoidal kinetic, characteristic of the cooperative binding of substrate on proteins with multiple polypeptide chains (Coton et al. 1998).

Biogenic amine synthesis can be performed by two types of enzymes with decarboxylation activity, i.e., biosynthetic or biodegradative enzymes, the latter are present at major level and are responsible for BA accumulation in foods. Under acidic stress conditions, the microorganisms activate a defense mechanism to survive by amino acid decarboxylation with consumption of protons and excretion of BAs and CO_2 (Fig. 1). In particular, the amino acid substrate enters into the cell

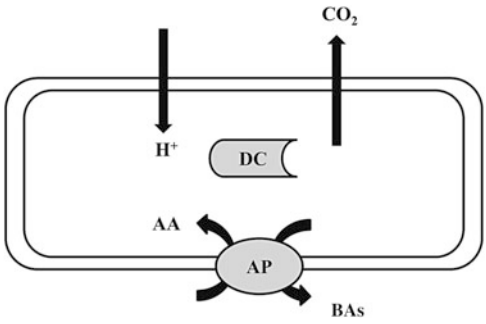

Fig. 1 Biogenic amine biosynthesis in microorganisms (Modified by EFSA 2011). Legend: *AP* antiporter, *BAs* biogenic amine, *AA* amino acid, *DC* decarboxylase enzyme

through the inner membrane antiporter protein with the subsequent excretion of BAs (EFSA 2011).

The catabolic pathway of BAs is generally regulated by oxidases classified as monoamine and diamine oxidases (MAO and DAO) and by specific amine *N*-methyltransferases (NMT) involved in BA removal (Tofalo et al. 2016).

Intestinal histamine absorption is regulated by MAO, released from enterocytes of the small bowel, and DAO which is synthesized in intestinal epithelial cells, as well as in renal proximal tubular cells. Thus, histamine can be metabolized by oxidative deamination or by ring methylation. In particular, DAO converts histamine into imidazole acetic acid, whereas NMT into *N*-imidazole acetic acid. These end products are excreted in the urine (FAO-WHO 2012; Maintz and Novak 2007).

If the amount of BAs ingested with food is too high or the metabolism is inadequate, due to genetic reasons regarding the detoxification enzymes or inhibitor effects of some medicines or alcohol, their presence (histamine above all) can result in cases of food intoxication or intolerance (Spano et al. 2010). The symptoms of histamine poisoning relate to effects on blood vessels and smooth muscles, including flushing, urticaria, headache, nasal secretion, bronchospasm, asthma, hypotension, hemoconcentration, eyelid edema, tachycardia, extrasystoles, and abdominal cramps but also oral burning sensation, peppery taste, and swelling of the tongue (Waldo et al. 2015; Wilson et al. 2012).

Most cases of histamine intoxication are self-limiting and patients recover within 1–2 days. When the symptoms are more severe, the first medical treatment consists of antihistamines, which can be H_2-blockers (i.e., ranitidine, cimetidine) or H_1-antagonists, such as diphenhydramine. In addition, corticosteroids can be used as adjuvant therapy. If histamine poisoning results in acute anaphylaxis, epinephrine is the first-line treatment to be immediately administered to patients (Waldo et al. 2015).

While some BAs such as cadaverine and putrescine are considered spoilage indicators, histamine formation has generally no relevance for flavor or taste of food; thus, this amine can reach high concentrations without organoleptic modification of food, causing potential intoxication for consumers. For this reason, foodstuffs containing high levels of some BAs, such as histamine or tyramine,

Table 1 Primers designed for PCR or qPCR detection of histamine-producing bacteria

	Primer name	5′-3′ sequence	Reference
Wine	JV16HC	AGATGGTATTGTTTCTTATG	Guo et al. (2015), Lucas et al. (2008), and Coton and Coton (2005)
	JV17HC	AGACCATACACCATAACCTT	
	CL1	CCWGGWAAWATWGGWAATGGWTA	
	CL2	GAWGCWGTWGTCATATTWATTTGWCC	
	106	AAYTCNTTYGAYTTYGARAARGARG	
	107	ATNGGNGANCCDATCATYTTRTGNCC	
	PHDC1	CCGTGCGGAAACAAAGAAT	
	PHDC2	CCGTGCGGAAACAAAGAAT	
	hdcAf	ATGAAGCCAGGACAAGTTGG	
	hdcAr	AATTGAGCCACCTGGAATTG	
	HDC1	ATGTCAGAGTTTGATAAAAAG	
	HDC2	TTAATAATTGATGTTTCCACC	
	HDC3[a]	GATGGTATTGTTTCKTATGA	
	HDC4[a]	CAAACACCAGCATCTTC	
Cheese	HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	de Las Rivas et al. (2006)
	HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA	
	STDEC-F	GAATTACCGATCTATGATGC	Rossi et al. (2011)
	STDEC-R	ACACCTTTGTTAGCACAAAC	
Fish	HDC-f	TCHATYARYAACTGYGGTGACTGGRG	Takahashi et al. (2003)
	HDC-r	CCCACAKCATBARWGGDGTRTGRCC	

Y = C or T; R = A or G; W = A or T; D = G, A, or T; N = A, C, G, or T
[a]Used for a multiplex PCR for simultaneous detection of Gram-positive histamine and tyramine-producing bacteria

don't produce off-flavors and consumers are unable to distinguish altered products (Russo et al. 2010).

The determination of BAs in food is necessary for their potential toxicity as well as the possibility of using them as food quality markers. Different methods have been developed for their detection and quantification in food: thin-layer chromatography, gas chromatography, capillary electrophoretic method, and high-performance liquid chromatography (Önal 2007) but also ELISA method (Kim et al. 2011) and electrochemical biosensors (Alonso-Lomillo et al. 2010).

The identification and/or quantification of histamine-producing bacteria in food require fast and reliable molecular tools, usually based on PCR methods targeting histidine decarboxylase gene. Actually, different culture-independent methods, such as real-time quantitative PCR, (qPCR) have been developed to detect histidine decarboxylase-positive bacteria in fishery products or during cheese-making and wine fermentation (Table 1). These methods contribute to a better understanding of the occurrence of histamine in foodstuffs, an information that is essential to reduce the unacceptable high frequency of histamine intoxication.

Table 2 Histamine content (mg/kg) in some food of animal origin and wine (mg/l)

Food	Histamine	References
Fish		
Indian mackerel	13.0	Humaid and Jamal (2014)
Fresh anchovies	69.0	Piersanti et al. (2014)
Fresh sardines	62.0	
Fresh tuna	478.0	Altieri et al. (2016)
Tuna hamburger	304.0	
Oil-preserved mackerel	>720.0	
Salted mackerel	68.0	Karmi (2014)
Salted tuna	146.0	
Cheese		
Parmigiano Reggiano	38.4	Spizzirri et al. (2013)
Grana Padano	23.9	
Provolone Valpadana	9.7	
Pecorino Crotonese	19.1	
Caciocavallo Silano	42.0	
Cabrales blue	957.6	Ladero et al. (2010)
Gouda	40.0	Ladero et al. (2009)
Gorgonzola	255.3	Mayer et al. (2010)
Roquefort	376.6	
Fermented sausages		
Catalão	6.1	Laranjo et al. (2016)
Salsichão	12.5	
Salami	192.0	Rauscher-Gabernig et al. (2009)
German types	6.2	De Mey et al. (2014)
French types	2.3	
Italian types	1.4	
Wine		
Red	2.8	Dang et al. (2013)
Red	2.2	Daniel et al. (2015)
Red	25.1	Meléndez et al. (2016)
White	12.5	
Rose	13.4	

In this chapter we focused our attention on the various categories of food with histamine and other BA contents including the measures of control and prevention. Histamine concentrations in some food of animal origin and wine are reported in Table 2.

2 Fish and Fishery Products

Scombroid poisoning or histamine fish poisoning is an allergy-like form of seafood-borne disease due to the consumption of fish containing high levels of histamine. The fish species include both scombroid such as tuna and mackerel and nonscombroid fish like sardine, anchovy, herring, mahimahi, and swordfish (Visciano et al. 2012). The presence of high concentrations of histamine can be a risk to consumers, especially if the amine-metabolizing capacity is saturated in individuals taking drugs that inhibit DAO or MAO as well as in patients with mastocytosis, tumor, or chronic myelocytic leukemia. Moreover, other BAs, such as putrescine, cadaverine, spermidine, spermine, and agmatine, can be formed in fishery products and inhibit intestinal histamine-metabolizing enzymes, thus enhancing its negative effects. Variation in individual sensitivity may be also the result of the interaction with other diet constituents, such as alcohol (EFSA 2015).

Scombroid poisoning is due to the consumption of fish species with high concentrations of free histidine in their flesh, which is decarboxylated to histamine under favorable conditions. Generally, histamine content is negligible in fish, usually below 0.1 mg/100 g (Auerswald et al. 2006), but after caught it can increase up to toxic concentrations for consumers. The European Union set limits of histamine in fish species belonging to the following families, Scombridae, Clupeidae, Engraulidae, Coryphaenidae, Pomatomidae, and Scomberesocidae, both fresh and treated by enzyme maturation in brine (Regulation 2073/2005/EC). In order to prepare a sampling plan to meet with an appropriate level for histamine in fish, it is necessary to define food safety criteria:

m = compliance criterion
M = acceptability criterion
n = number of units comprising the sample
c = number of sample units giving values between m and M

Based on nine samples, the maximum average histamine content is 100 mg/kg; no more than two samples can have histamine concentrations between 100 and 200 mg/kg; no sample can have histamine content higher than 200 mg/kg. Recently Regulation 1019/2013/EU established that when a single sample taken at retail level exceeds M, the whole batch should be deemed unsafe (Visciano et al. 2014).

Even if these regulatory limits have to be met only for the abovementioned fish families, scombroid poisoning symptoms have been reported also after the consumption of other species of fish containing low histidine and histamine levels. It has been supposed that endogenous rather than ingested histamine could be released by mast cells in human body, but the mechanism is still unknown (FAO-WHO 2012).

The formation of high levels of histamine in fish is associated with some conditions: (1) natural microbiota depending on habitat, (2) temperature of storage after caught, and (3) inadequate handling onboard the harvest vessel. Live fish contain bacteria on the gills, on external surfaces, and in the gut, but once the fish

died, the defense mechanisms no longer inhibit bacterial growth, and the loins become the main focus for microorganism proliferation. The marine habitats vary significantly and can affect the initial contamination of fishery products. In particular, seawater temperatures depend on many factors, such as latitude, depth, and oceanographic currents, so that also initial fishery product temperatures vary from −4°C (polar waters) to 35°C (equatorial waters). The general approach after the caught of fish relies on achieving the temperature of melting ice as soon as possible even if some factors can influence the speed of reaching this temperature, like the size and the initial temperature of fish, the ratio of fish to ice, and the completeness of cavity icing (EFSA 2015). According to Regulation 853/2004/EC, in vessels equipped for chilling fishery products, tanks must incorporate devices for achieving a uniform temperature of mix of fish and clean seawater, not more than 3°C 6 h and not more than 0°C 16 h after loading. However, in some harvesting practices, such as long-lining, many hours may pass before the fish is brought onboard the vessel. The European legislation specifies that when chilling is not possible onboard the vessel, fresh fishery products must undergo chilling as soon as possible after landing.

Some operations such as evisceration and removal of the gills may reduce but not eliminate the number of histamine-forming bacteria and must be carried out hygienically (FDA 2011). However defective handling techniques due to poor hygienic conditions can result in the presence of high levels of histamine in fish.

Histidine decarboxylation has been observed in different bacterial species including *Morganella morganii*, *Proteus vulgaris*, *Hafnia alvei*, *Photobacterium phosphoreum*, *Photobacterium psychrotolerans*, *Citrobacter freundii*, and *Enterobacter aerogenes* (Ladero et al. 2010). As most of these bacteria are mesophilic, histamine formation can be prevented if fish is stored at refrigeration temperatures even if some of them (i.e., *Morganella psychrotolerans* and *P. phosphoreum*) have been reported as significant producers of histamine at temperatures of 0–5°C. The activity of histidine decarboxylase is temperature independent and once formed during storage at high temperatures remains active also at 0–5°C or below (EFSA 2015). Therefore, histamine formation is strictly dependent on temperature/time abuse; in particular it is greater at high abusive temperatures (21.1°C or higher) than at moderate abusive temperatures (7.2°C) and is particularly rapid at temperatures near 32.2°C (FDA 2011). During the merchandising chain, high-temperature abuse together with mishandling can enhance its production. So, the cold chain control coupled with efficient and hygienic handling of fishery products represents the main risk management strategy for minimizing/inhibiting histamine formation in fresh fish.

Since histamine is not uniformly distributed in all parts of fish, the dose-response relationship in consumers can be different even if they eat the same fish (Lehane and Olley 2000). For this reason, FDA (2011) set a guidance level of 50 mg/kg of histamine in the edible portion of fish because this value can be found in one section of a single fish but there is also the possibility that other sections may exceed 500 mg/kg.

In addition to histamine, other BAs can occur in fish and fishery products indicating the quality of their flesh. This parameter has been defined Biogenic Amine Index (BAI) and expressed in different ways such as the sum of cadaverine and putrescine according to Stede and Stockemer (1981) or the sum of histamine + putrescine + cadaverine + tyramine (Veciana-Nogués et al. 1997).

Another BAI has been described by Mietz and Karmas (1981) as the sum of putrescine + cadaverine and histamine/1 + spermidine + spermine. Nevertheless, it can be resumed that fish spoilage is a very complex event and chemical analysis alone is not sufficient to detect the quality of fish (Prester 2011).

The fishery products can be processed by brining, salting, smoking, drying, fermenting, and pickling, and also in these cases, histamine can be formed. Food safety criteria have been set for fish treated by enzyme maturation in brine by the European legislation corresponding to 200 mg/kg as *m* and 400 mg/kg, as *M*. More recently, the Regulation 1019/2013/EU fixed a maximum value of 400 mg/kg in fish sauce produced by fermentation, to be met by only one sample because it is a liquid fishery product and histamine can be expected to be evenly distributed.

Histamine is very heat stable and once formed will not be destroyed even by heat treatment at high temperature such as autoclaving (121°C for 15–20 min) (EFSA 2015). The presence of histamine in canned fish is due to use of poor quality raw material in which the amine has been already formed. Moreover, histamine accumulation can occur also when frozen fish are thawed and kept for long periods at room temperature before further processing (Prester 2011). So, temperature/time abuse during the different industrial processes can enhance the growth and the proliferation of histidine decarboxylase-positive bacteria.

Unhygienic practices and insufficient refrigeration can cause histamine formation and subsequently outbreaks of histamine poisoning. The application of good manufacturing practices according to HACCP (hazard analysis and critical control point) system can reduce BA levels, histamine included, during fish caught, storage, and further processing.

3 Cheese

Among the fermented foods, cheese is one of the most commonly associated with high BA concentrations, as a result of microorganism activity, with histamine, tyramine, putrescine, cadaverine, and phenylethylamine being the principal ones. The EFSA (2011) reported that after fish, cheese – including products made from raw as well as pasteurized milk – is involved in histamine poisoning. Gouda, Swiss cheese, Cheddar, Gruyère, and Cheshire have been associated with histamine poisoning in patients subject to isoniazid therapy. An outbreak related to grated cheese was described in Spain. The histamine concentrations in cheeses that were implicated in such outbreak ranged between 850 and 1,870 mg/kg. The total BA and in particular histamine concentrations in cheeses with similar microbiological profiles may differ according to many factors such as the presence of decarboxylase-positive bacteria, level of proteolysis (availability of substrate),

pH, salt-in-moisture level, and temperature. Some microbial groups, which are part of milk and cheese microbiota, possess histidine decarboxylase activity and hence are potential histamine producers. Besides bacteria, also yeasts are potential producers of putrescine and cadaverine, and histamine formation capacity has been reported in *Debaryomyces hansenii* (Gardini et al. 2006). The main producers of histamine in cheese are LAB that can be part of the starter cultures, but also of the non starter lactic acid bacteria (NSLAB) that are generally present in increasing number throughout cheese ripening (Schirone et al. 2012). Moreover, NSLAB can grow under selective conditions of cheese ripening and contribute to the formation of small peptides and amino acid precursors of BAs. The amine production by the different bacteria can widely range; however, no correlation has been found between histamine content and the total bacterial count. Some cheeses can contain more than 1,000 mg/kg of this amine. Histamine occurrence does not seem to be associated with a specific milk (goat, cow, and sheep milk) used in cheese production but it depends above all on the microbiological quality of raw material (Ladero et al. 2008). However, some studies found that ovine cheese was characterized by a remarkably higher accumulation of BAs than bovine cheese. Even if low concentrations of histidine are present in milk, the proteolysis during cheese ripening can liberate a large amount of such precursor. An important event that can occur during ripening of cheese is the change of proteins. The proteins (overall casein) retained in curd are initially degraded to large peptides, which in turn are degraded to small peptides and, finally, to free amino acids. Such process is guaranteed by proteases or peptidases produced by microorganisms, present in milk, and/or in rennet. Due to these activities, the availability of precursors is a central problem in BA accumulation (Lanciotti et al. 2007). For this reason, the enhancement of proteolysis during cheese ripening by addition of proteolytic enzymes can increase the concentration of BAs. High histamine levels can be detected in blue cheeses, as reported by Ladero et al. (2008) due to the presence of fungi with a strong proteolytic activity.

There is a general consensus that BA accumulation in cheese is influenced by the microbial quality of raw milk, the adopted sanitation procedures, the use of starter cultures, and the condition and time of the ripening process. In particular, the microbial population of raw milk can influence BA presence in cheese, even when thermal treatment has been applied (Lanciotti et al. 2007). Pasteurization is the most common milk treatment used during cheese-making aimed at reducing the number of pathogenic and spoilage microorganisms, and many studies showed that its application is able to reduce BA concentration (Ladero et al. 2010). However, high BA levels in cheese produced using pasteurized milk and natural milk culture have been found (Marino et al. 2008). The thermal treatment of milk seems to be not enough by itself to reduce the counts of decarboxylase-positive bacteria in cheese. Metabolically active BA producers can be found in pasteurized milk owing to their resistance to thermal treatment (Ladero et al. 2011). The ripening of cheese is another factor strongly affecting the levels of BAs in cheese that are, in general, much higher than those detected in unripened cheeses. The BA content can be from

10 to 2,000 times higher, depending also on the intensity of the ripening process (Mayer et al. 2010).

Some cheese post-ripening processes, such as cutting, slicing, or grating, have been found to increase the presence of histamine probably for a contamination with histamine producers (Ladero et al. 2009).

The prevention of BA formation in cheese can be achieved by using temperature control, high-quality raw material, good manufacturing practices, nonamine-producing (amine-negative) or amine-oxidizing starter cultures for fermentation, enzymes able to oxidize amines, use of microbial modeling, packaging techniques, high hydrostatic pressure, irradiation, and food additives. Emerging approaches to control histamine production involve the combined effect of two existing methods, such as high hydrostatic pressure and the use of decarboxylase-negative starters. In addition, the detection of histamine-producing bacteria in the first phases of cheese-making should allow to foresee the evolution of histamine content.

4 Dry Fermented Sausages

In fresh meat the only BAs are spermidine and spermine and to a lesser extent putrescine, whereas high concentrations of these amines can be attributed to microbial growth. On the contrary, fermented meat products show a wide variability in BA content. In particular, the fermented dry sausages are still manufactured with traditional technologies, and for this reason they are characterized by spontaneous fermentation without selected starter cultures. Many factors (such as pH, water activity, redox potential, NaCl, sausage diameter, etc.) can have an important effect on the production of BAs by microorganisms with a different ability in synthesizing decarboxylases. These microorganisms are mainly LAB, species belonging to the genera *Staphylococcus* and *Kocuria* (coagulase-negative cocci, CNC), yeasts, and molds. In the meat industry, the use of starter cultures containing mixtures of LAB and CNC that do not form BAs and are competitive in suppressing the growth of wild amine-producing microbiota can be a valid approach to guarantee the food safety and to standardize the product properties (Suzzi and Gardini 2003).

The microbiota of dry fermented sausages is closely related to the manufacturing process and in particular to the ripening time. Sausages with a short ripening have more lactobacilli instead of CNC that are more representative in sausages with a long ripening. For this reason, a wide variability in BA content has been observed, even in products with comparable chemical and microbial profiles. The proteolysis of meat sarcoplasmic and myofibrillar proteins is one of the main biochemical phenomena occurring during the ripening of fermented sausages, determined by both endogenous muscle and microbial enzymes. The quantities of BAs show a large variability among the types of product and process with tyramine being the most abundant and frequent. Histamine concentration in dry fermented sausages can vary largely, generally detected at low levels in many samples but in some others reaching values of health concern well above 100 mg/kg (Bover-Cid et al. 2001).

Papavergou (2011) did not observe histidine decarboxylase activity in lactobacilli isolated from sausage contrary to what was reported in other studies (Maijala 1994).

The control of microorganisms able to produce histamine or in general BAs could be carried out by the use of starter cultures (Latorre-Moratalla et al. 2012). *Lactobacillus sakei* strains, characterized to be amine-negative producers and particularly useful for sausage fermentation, were found to reduce the overall amine accumulation. However, the starter cultures are not always able to control the decarboxylase-positive strains, depending on raw meat microbiological quality and the characteristics of natural microbiota, in particular amine-positive NSLAB that are often responsible for BA formation in fermented sausages. In French sausages, high concentration of histamine was found in industrial products added with starter cultures rather than in artisanal sausages. The effectiveness of starter cultures is affected by raw material and optimization of the technological conditions that can favor proper implantation and development of the starter cultures.

All the parameters that affect microbial growth and metabolism during fermentation and storage of fermented sausages have an important role on BA accumulation, for example, the variation in the quantity of water and in the salt/water ratio. *Lactobacillus bulgaricus* (now *L. delbrueckii* subsp. *bulgaricus*) strain reduced amine production by increasing salt concentration in the medium from 0 to 6% (Chander et al. 1989). Other authors (Chin and Koehler 1986) demonstrated that NaCl concentration ranging from 3.5 to 5.5% could inhibit histamine production. In general, the factors affecting the activity of the decarboxylating enzymes appear to be more important than the precursor availability. High temperature, high pH, and low salt content can accelerate the amino acid accumulation and, hence, stimulate the amine formation, during fermentation and ripening processes. In order to reduce or prevent histamine formation, handling and processing under hygienic conditions, histidine decarboxylase-negative starter cultures, diamine oxidative starter cultures, high-pressure processing, low-dose gamma irradiation, vacuum or modified atmospheres, and modeling approaches have been proposed (Naila et al. 2010). In addition, ingredients and additives used in dry sausage formulation are important factors to modulate the BA formation.

5 Wine

Wine fermentation is a complex process influenced mainly by yeasts and LAB. Two successive fermentations – an alcoholic fermentation (AF) led by the wine yeast *Saccharomyces cerevisiae*, which allows the conversion of grape sugar to ethanol and CO_2, and a subsequent malolactic fermentation (MLF) carried out, in most red and some white wines, by LAB of the genera *Oenococcus*, *Lactobacillus*, and *Pediococcus* – are required. Malolactic fermentation is useful not only to deacidify the medium by the conversion of L-malic acid to L-lactic acid but also to ensure a certain degree of microbial stability to the wine and modify the wine sensory characteristics through the production of secondary bacterial metabolites (Lonvaud-Funel 1999). However, spoilage microorganisms can grow and produce

compounds which can negatively influence the final quality of the wine. Therefore, wines can contain toxic or even carcinogenic molecules, i.e., BAs, ochratoxin A, and ethyl carbamate, deriving from the microbial metabolism.

Biogenic amines are associated with wine allergies. However, there are studies showing that no relationship exists between the oral ingestion of BAs and wine intolerance (Jansen et al. 2003).

Biogenic amines can be normal constituents of grapes, and their amount depends on grape variety, region, soil type and composition, fertilization and climatic conditions during grape ripening, and degree of maturation (García-Villar et al. 2007). In addition to those already present in grapes, other BAs can be formed during the wine-making. Oenological practices and in particular the use of the commercial pectolytic enzyme preparation can promote BA accumulation in wines as well as hot maceration, and reductive fermentation conditions could result in higher BA concentrations (Ancín-Azpilicueta et al. 2008). The main BAs found in wines are histamine, tyramine, putrescine, cadaverine, and phenylethylamine (Beneduce et al. 2010; Coton et al. 1998). It is known that BAs can affect human health and cause the low quality of wines; however, their production in wines is quite inevitable. Their formation mainly relies on three conditions: availability of the precursor amino acid, presence of microorganisms with decarboxylase activity, and conditions which permit both microbial growth and the expression of the decarboxylase activity (Ten Brink et al. 1990).

There are conflicting opinions regarding the formation of BAs during AF. However, it is generally agreed that BA concentration is low at the end of AF and increases during MLF, suggesting that their production is related to bacterial metabolism. Some authors demonstrated that strains of the main malolactic species such as *Oenococcus oeni*, as well as *Lactobacillus* and *Leuconostoc* genera, showed a decarboxylation activity (Moreno-Arribas et al. 2003).

Histamine is considered the most important cause of wine intolerance (Konakovsky et al. 2011). Yeast, acetic acid bacteria, and LAB generally present in wine are all considered possible histamine producers, although LAB are reported to be the main bacteria responsible for histamine production in wine. In particular, Landete et al. (2005) demonstrated that *Lactobacillus hilgardii* and *Pediococcus parvulus* are the histamine producers principally responsible for the high histamine concentrations in wine; in fact they are able to produce histamine at concentration higher than 200 mg/l. Other strains able to contribute to histamine in wine are *Lactobacillus mali* and *Leuconostoc mesenteroides*. Also *O. oeni* is considered a histamine producer even if at low levels. It is known that the production capacity of histamine depends on each strain within the same species (Lonvaud-Funel 2001) and that histamine concentration increases especially when MLF takes place without the addition of selected bacteria (López et al. 2008).

The ability of these strains to produce histamine is due to the presence of bacterial histidine decarboxylase (HDC; E.C. 4.1.1.22) activity. Histidine decarboxylase was firstly isolated by Lonvaud-Funel and Joyeux in 1994 from *O. oeni* 9204. It is a polypeptide of 315 amino acids with α- and β-subunits with an optimum pH of 4.8. It is dependent on pyridoxal 5′-phosphate cofactor. However,

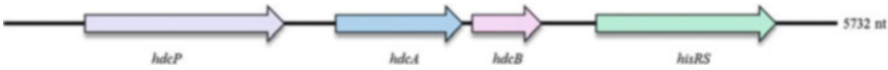

Fig. 2 Schematic representation of the genetic organization of *hdcA* locus of *L. hilgardii* 0006. Modified from Patrick et al. (2005). *hdcA* encodes for histidine decarboxylase, *hdcP* for a histidine/histamine exchanger, *hisRS* gene for a histidyl-tRNA synthetase, *hdcB* for a protein of unknown function

some HDCs of bacterial origin use pyruvoyl cofactor, which is part of the active site and is located at the amino terminus of the alpha chain, at the break between the β- and α-chains. The pyruvoyl cofactor facilitates decarboxylation via a Schiff base mechanism resembling that of the more common PLP-dependent decarboxylases. This covalent intermediate allows for resonance stabilization, facilitating the decarboxylation step. At low histidine concentrations, HDC has a low substrate affinity, and histamine acts as a competitive inhibitor of the antiport histidine/histamine at the cell membrane and decreases the HDC activity. In a wine *L. hilgardii* strain demonstrated the presence of an 80 kb plasmid on which the decarboxylase gene was located as part of a four-gene cluster, *hdc*P, *hdc*A, *his*RS, and *hdc*B, coding for a histidine/histamine exchanger, a HDC, a histidyl-tRNA synthetase, and an unknown product, respectively (Fig. 2). Probably this plasmid could be transferred horizontally among bacteria explaining the random distribution of HDC-positive bacteria. Recently, Landete et al. (2006) investigated the effects of histamine, histidine, pyridoxal 5′-phosphate, and growth phase on histamine production by LAB isolated from wine. They observed that histidine (at 1 or 2 g/l) and pyridoxal 5′-phosphate (at 0.5 g/l) induce the expression of *hdc* gene, while histamine (at 1 or 2 g/l) causes a decrease in the expression of this gene. In addition the expression of this gene is also mediated by the bacterial growth phase. In particular its expression is the highest in the exponential growth phase because histidine decarboxylation generates metabolic energy while it diminishes during the stationary growth phase, during which growth and cell division are reduced.

In wine HDC activity is favored at pH 3.5 and by ethanol concentrations up to 10% since histidine transport inside the cells is increased due to the fluidification of the cell membrane by ethanol (Lonvaud-Funel and Joyeux 1994). Ethanol concentrations, 12% or more, can reduce the HDC activity since the physicochemical properties of the membrane are altered causing reduction of histidine transport (Rollan et al. 1995).

The detection of BA-producing bacteria in fermented beverages is mainly based on PCR approaches. Generally, these methods detect bacteria harboring BA-producing pathway genes, since a relationship between the presence of the gene encoding the decarboxylase and the capacity to synthesize BAs has been reported by several authors (Landete et al. 2005; Lucas et al. 2005). In particular, some conserved regions in *hdc* gene have been detected in *Lactobacillus* 30a, *Clostridium perfringens*, *Lactobacillus buchneri*, and *Micrococcus* sp. and used to design specific primer sets (Table 1). Recently, Marcobal et al. (2005) developed a multiplex PCR method for the simultaneous detection of histamine-, tyramine-,

and putrescine-producing LAB in wine and cider. Also real-time quantitative PCR protocols have been developed for detecting histamine-producing LAB in wine (Lucas et al. 2008) successfully used to monitor the different steps of wine fermentation (Nannelli et al. 2008).

Nowadays researchers are looking for solutions aimed at the reduction of BA content in wine. A possibility is the inoculation with starter cultures unable to produce BAs (Spano et al. 2010). However, reports on the potential role of these starters in the elimination/degradation of BAs in wines are lacking.

Many authors observed that BA (i.e., histamine and tyramine) concentrations can decrease during wine storage and aging (Jiménez Moreno et al. 2003). This might be due to the action of amine oxidase enzymes present in the wines (Ancín-Azpilicueta et al. 2008). Recently the potential for BA breakdown has been proved to be a characteristic of the genera *Lactobacillus* and *Pediococcus* (Leuschner et al. 1998). The ability to degrade histamine has been demonstrated to be low within the natural population of *O. oeni* isolated from wines.

However, a definitive solution doesn't exist since an effective control of BA content may require a combination of several factors. For instance, the use of starter cultures together with high-quality raw materials and appropriate wine-making practices could represent the best way of making wines with reduced BA-associated health risks. In 2011, the International Organization of Vine and Wine (OIV) published the "OIV code of good vitivinicultural practices" in order to reduce the presence of BAs in wine-based products. The main actions proposed concerned both vineyards (kind of soil and agronomic practices) and cellars (hygiene practices, use of selected yeasts and LAB strains during AF and MLF, respectively).

6 Other Foods

Many studies reported histamine content in different kinds of food of vegetable origin (Table 3). In particular histamine occurrence has been described in non-fermented and fermented soybean products at different concentrations, but the lack of information about its presence may increase the risk arising from the consumption of these products (Toro-Funes et al. 2015).

Soybean is used worldwide in a variety of traditional fermented products such as miso, natto, tempeh, soy sauce, tofu, and sufu. In particular, sufu is a traditional soft cream cheese-like fermented soybean food produced in China and made in three steps from tofu (Guan et al. 2013).

Some authors reported histamine levels also in various fresh vegetables such as spinach, tomato, broad bean, and broccoli (Moret et al. 2005).

Table 3 Histamine content (mg/kg) in food of vegetable origin

Food	Histamine	References
Soy sauce	nd-592	Yongmei et al. (2009)
Sufu	730.0	Toro-Funes et al. (2015)
Miso	nd-221	Kung et al. (2007)
Natto	nd-457.0	Tsai et al. (2007)
Soy milk	17.5	Saaid et al. (2009)
Tempeh	4.1	
Tofu	3.5–5.8	Byun et al. (2013)
Soybean paste	51.2	Toro-Funes et al. (2015)
Tamari	57.65	
Tomato pasta	2.0–10.1	Kalač et al. (2002)
Ketchup	2.0–18.0	
Spinach purée	2.1–9.8	
Green pea	2.3–3.8	

7 Conclusions

The toxicity of histamine individually or combined with other BAs is a relevant aspect for public health, and the actual knowledge need more information about the preventive measures able to reduce the presence and accumulation of this compound in foodstuffs. Among groups of foods and within each group, there can be a wide variability in the amounts of BAs, but consumer exposure is also affected by their habits and food preference. Therefore, histamine dietary intake can be particularly high following the consumption of one or more food items containing high concentrations of this amine during the same meal.

Further identification of data able to support a quantitative risk assessment for consumers represents an important step in order to guarantee food safety.

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Regulation of the Cardiovascular System by Histamine

Yuichi Hattori, Kohshi Hattori, and Naoyuki Matsuda

Contents

Abstract

Histamine mediates a wide range of cellular responses, including allergic and inflammatory reactions, gastric acid secretion, and neurotransmission in the central nervous system. Histamine also exerts a series of actions upon the cardiovascular system but may not normally play a significant role in regulating cardiovascular function. During tissue injury, inflammation, and allergic responses, mast cells (or non-mast cells) within the tissues can release large amounts of histamine that leads to noticeable cardiovascular effects. Owing to intensive research during several decades, the distribution, function, and

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pathophysiological role of cardiovascular H_1- and H_2-receptors has become recognized adequately. Besides the recognized H_1- and H_2-receptor-mediated cardiovascular responses, novel roles of H_3- and H_4-receptors in cardiovascular physiology and pathophysiology have been identified over the last decade. In this review, we describe recent advances in our understanding of cardiovascular function and dysfunction mediated by histamine receptors, including H_3- and H_4-receptors, their potential mechanisms of action, and their pathological significance.

Keywords
Heart • H_3-receptors • H_4-receptors • Vasculature

1 Introduction

A wide range of responses to histamine throughout the cardiovascular system have been appreciated for more than 100 years since the seminal work of Dale and Laidlaw (1910). Some of the cardiovascular responses to histamine have had a key role in the historical development of the concept of histamine receptors and in the identification of histamine receptor antagonists. Histamine is synthesized in several cell types, including mast cells, and histamine synthesis is mediated by the enzyme, L-histidine decarboxylase (HDC), which catalyzes decarboxylation of L-histidine (Garbarg et al. 1974). Each of the histamine receptors produces a functional response, but their signaling mechanisms are different. Histamine exerts its biological effects by binding and activating four distinct types of G protein-coupled receptors, designated as H_1, H_2, H_3, and H_4 (Parsons and Ganellin 2006). H_1-receptors couple to $G_{q/11}$ stimulating phospholipase C (PLC), whereas H_2-receptors interact with G_s to activate adenylate cyclase (Hill et al. 1997). H_3- and H_4-receptors couple to G_i proteins to inhibit adenylate cyclase and to stimulate MAPK in the case of H_3-receptors (Drutel et al. 2001; Liu et al. 2001a; Hofstra et al. 2003). The identification of H_3- and H_4-receptors more than a dozen years ago revived the interest in histamine research and exposed attractive perspectives for the potential therapeutic exploitation of these new drug targets in neurological and immunological disorders (Tiligada et al. 2009, 2011; Zampeli and Tiligada 2009).

A growing body of evidence is accumulating to show that histamine exerts responses in most parts of the cardiovascular system mediated by H_1- and H_2-receptors, although an important feature of the cardiovascular responses to histamine is variation between species. The histamine responses on the cardiovascular system may be of special importance in certain pathological aspects suggestive of histamine release, such as immediate hypersensitivity and septic shock. For instance, endotoxemia causes a significant increase in histamine production through induction of HDC and upregulates gene expression of histamine receptors in cardiovascular tissues, which may contribute to the hemodynamic derangement associated with this pathological disorder (Matsuda et al. 2002, 2003, 2004a). More importantly, besides the recognized H_1- and H_2-receptor-mediated cardiovascular

responses, novel roles of H_3- and H_4-receptors in cardiovascular physiology and pathophysiology have been identified over the last decade.

The large body of literature on the actions of histamine on the cardiovascular system has been reviewed in extenso in a previous volume of this series (Levi et al. 1991) and elsewhere (Levi and Allan 1980; Trzeciakowski and Levi 1981; Levi et al. 1982; McNeill 1984; Hattori 1999). We underline our current understanding of the principal cardiovascular actions of histamine in this chapter. The recent developments on this subject will be also considered here with special focus on H_3- and H_4-receptors.

2 Histamine Receptor Expression in the Heart

Histamine is substantially present in the heart of most animal species, including humans (Bristow et al. 1982; Wolff and Levi 1986). The distribution of histamine within the area of the heart parallels that of mast cells, indicating that mast cells are a major site of storage of histamine in the heart (Guidotti et al. 1967). However, ample evidence has been provided for the presence of appreciable amount of non-mast cell cardiac histamine (Riley and West 1955; Johnson 1970; Harvey 1978). The content of histamine released from cardiac storage sites by anaphylaxis or a variety of chemicals has been shown to be sufficient to cause significant changes in cardiac functions (Pöch and Kukovetz 1967; Levi 1972; Levi and Allan 1980). Although it is difficult to assign a physiological role for histamine in the heart, its release from cardiac stores and its subsequent actions on the heart may be of importance in certain pathological aspects suggestive of histamine release (Levi 1988).

The primary and direct actions of histamine on the heart are characterized by an increase in sinus rate and ventricular automaticity, a decrease in atrioventricular (AV) conduction velocity, and an increase in force of contraction (Levi et al. 1991; Hattori 1999). The positive chronotropic and negative dromotropic effects of histamine invariably result from activation of H_2- and H_1-receptors, respectively. However, a marked species difference has been found in the subtype of histamine receptors mediating a positive inotropic effect. In addition, different receptor subtypes mediate the positive inotropic effect in different parts of the heart within the same species (Hattori 1999). In the guinea pig heart, only H_1-receptors are responsible for the positive inotropic effect of histamine in left atrium, whereas H_2-receptors predominantly mediate its positive inotropic effect in ventricle (Steinberg and Holland 1975; Verma and McNeill 1977; Hattori et al. 1994). In contrast, in the rabbit heart, H_2-receptors mediate the positive inotropic effect of histamine in left atrium, whereas H_1-receptors are predominantly involved in its positive inotropic effect in ventricle (Hattori et al. 1988, 1990, 1994). In yet another mode, the positive inotropic response of the rat heart to histamine appears to be totally attributed to release of endogenous catecholamine, since the increase in contractions of isolated rat myocardial tissues elicited only by very high

concentrations of histamine could be blocked by propranolol or reserpine pretreatment (Laher and McNeill 1980).

Radioligand-binding studies indicate that the species difference in the subtype of histamine receptors predominantly mediating the positive inotropic effect of histamine is unlikely to result from a difference in distribution of H_1- and H_2-receptors. The [^{3}H]mepyramine binding data have denoted the significant presence of H_1-receptors in guinea pig ventricular myocardium (Chang et al. 1979; Bennardini et al. 1984), despite of the less contribution of H_1-receptors to the positive inotropic effect of histamine in this tissue. Our radioligand-binding assay with [^{3}H]mepyramine has shown that the density of H_1-receptors in guinea pig is higher than in rabbit ventricular myocardium (Hattori et al. 1994). Conversely, the density of H_2-receptors estimated with [^{3}H]tiotidine is higher in rabbit than in guinea pig ventricular myocardium (Hattori et al. 1994). Given the functional studies showing that the positive inotropic responses to histamine are dominated by the H_1-receptor-mediated effect in rabbit and by the H_2-receptor-mediated effect in guinea pig ventricular myocardium, there is a dissociation of the functional response from the binding characteristics with respect to histamine receptor subtypes in ventricles of these two species. Moreover, we have found specific binding of [^{3}H]tiotidine, in addition to that of [^{3}H]mepyramine, to membranes from guinea pig left atria in which histamine exert a positive inotropic effect mediated exclusively by H_1-receptors (Hattori et al. 1991). These results suggest that the coupling between each of histamine receptor subtypes and the signal transduction mechanisms mediating the positive inotropic effect may vary considerably among species, depending on the regions of the heart. Caution is required, however, in interpretation of estimation of histamine receptor distribution using radioligand-binding methods, because the use of the radioligands to label histamine receptors could be hampered by high nonspecific binding and/or significant binding to secondary non-histamine-receptor sites (Chang et al. 1979; Hill and Young 1980; Foreman et al. 1985; Rising and Norris 1985; Liu et al. 1992).

We have analyzed gene and protein expression levels of H_1- and H_2-receptors in the hearts of mammals using Northern and Western blotting techniques, respectively (Matsuda et al. 2004b). Western blot analysis has been conducted using H_1- and H_2-receptor antibodies, both of which are widely available to the histamine research community, but it is warned that anti-peptide antibodies using peptide comprising sequences deduced from histamine receptor sequences have to be carefully evaluated for their ability to recognize the holo-protein (Seifert et al. 2013). We have found that the expression levels of H_1- and H_2-receptor proteins in myocardial tissues from mammalian species employed correlate with their mRNA expression levels, implying that the expression of H_1- and H_2-receptors in mammalian hearts is regulated in a transcriptional manner. In guinea pigs, both Northern and Western blot analysis have shown that the H_1-receptor is expressed in the heart at a relatively high level, mainly in the atrium and less in the ventricle, when compared with its abundant expression in the brain. In contrast, the H_2-receptor is abundant in the ventricle and slightly expressed in the atrium. On the other hand, in rabbits, the H_2-receptor is a predominant histamine receptor subtype in the atrium, with much less H_1-receptor expression, whereas the

ventricle expressed an abundance of H_1-receptors and much less H_2-receptors. These findings could well explain those of previous pharmacological studies, clearly demonstrating that the positive inotropic responses of the heart to histamine depend on the expression level of H_1- and H_2-receptors. In rats, H_2-receptors have been found to be present at a rather low level both in the atrium and ventricle; in contrast, expression of H_1-receptors is significant in both tissues. As stated above, a weak positive inotropic response caused by histamine only at very high concentrations is likely to result from catecholamine release. It is thus conceivable that the H_1-receptor may be weakly coupled to its signal transduction pathways responsible for inotropy in the rat heart.

In human atrial and ventricular muscles, histamine produces a positive inotropic effect exclusively mediated by H_2-receptors (Eckel et al. 1982; Levi et al. 1991; Du et al. 1993). Using Northern and Western blot analysis, we have determined the gene and protein expression levels of H_1- and H_2-receptors in right atrial tissues from patients undergoing aortacoronary bypass surgery and in left ventricular tissues from patients with moderate heart failure during surgical resection of left ventricular aneurysms (Matsuda et al. 2004b). In human atrial and ventricular tissues, it has been shown that H_1-receptor expression is less abundant, whereas H_2-receptors are richly expressed at both mRNA and protein levels. These results are in good agreement with the above functional reports. Thus, H_2-receptors are present as the predominant histamine receptor subtype in the human heart.

Immunohistochemical studies have demonstrated the presence of both H_1- and H_2-receptors in the sinoatrial (SA) and AV nodes of the guinea pig heart (Matsuda et al. 2004b). Although it is not clear what role, if any, is played by the H_1-receptor present in the SA node, there is firm evidence that H_2-receptors which are present in the SA node primarily mediate the histamine-induced increase in sinus rate (Levi et al. 1991; Hattori 1999). In the guinea pig heart, histamine is known to impair AV conduction mediated by H_1-receptors (Levi 1972; Levi et al. 1975). On the other hand, histamine may also have an ability to enhance automaticity in the AV node. Electrophysiological studies using AV node preparations using the rabbit and guinea pigs have indicated that H_2-receptors accelerating an increase in automaticity are likely to exist in the AV node (Borchard and Hafner 1986; Sanchez-Chapula and Elizalde 1987). Accordingly, these pharmacological results can be fully supported by immunohistochemistry demonstrating the presence of both H_1- and H_2-receptors in the AV conduction system.

3 Protective Role of H_3- and H_4-Receptors Against Cardiac Dysfunction in Myocardial Ischemia

Myocardial infarction is often accompanied by severe arrhythmias with high morbidity and mortality. Sympathetic overactivity with excessive noradrenaline release is clinically recognized as a prominent cause of arrhythmic cardiac dysfunction in myocardial ischemia (Braunwald and Sobel 1988; Dart and Du 1993; Benedict et al. 1996). Calcium-dependent exocytotic and nonexocytotic carrier-mediated efflux are the major mechanisms of noradrenaline release from

sympathetic nerve endings. The latter is independent of extracellular calcium and accounts for the release of noradrenaline induced by indirectly acting sympathomimetics, such as tyramine, and by low energy states, such as anoxia and cyanide intoxication (Haass et al. 1989). In physiological conditions and short-term myocardial ischemia, noradrenaline release is exocytotic and is thus dependent on a rise in axoplasmic calcium ion concentrations (Kübler and Strasser 1994). In protracted myocardial ischemia, however, noradrenaline is abundantly carried out of sympathetic nerve terminals by the noradrenaline transporter functioning in a reversed outward mode; this nonvesicular noradrenaline release process is known as carrier mediated (Levi and Smith 2000). A massive efflux of noradrenaline via a reversal of the noradrenaline transporter, which results from compensatory activation of neuronal Na^+/H^+ exchanger (NHE) by axoplasmic acidification, could trigger severe arrhythmias (Levi and Smith 2000). Accordingly, negative modulation of noradrenaline release from cardiac sympathetic nerves is a crucial mechanism of cardiac protection.

Histamine H_3-receptors are one of several classes of prejunctional heteroinhibitory receptors. In the hearts of mammals, including human, sympathetic nerve endings express H_3-receptors (Endou et al. 1994; Imamura et al. 1995; Levi and Smith 2000). H_3-receptors can inhibit sympathetic neurotransmission in the heart, which has been demonstrated by the findings that selective H_3-receptor agonists such as (R)α-methylhistamine and imetit decrease the inotropic and chronotropic responses to cardiac sympathetic nerve stimulation (Luo et al. 1991; Endou et al. 1994; Mazenot et al. 1999). Histamine appears to be locally released from mast cells in myocardial ischemia (Imamura et al. 1994; Hatta et al. 1997), and H_3-receptors are plausibly fully activated under ischemic conditions. Indeed, the selective H_3-receptor antagonist thioperamide could double the overflow of noradrenaline at reperfusion in the early phase of myocardial ischemia where noradrenaline exocytosis is enhanced (Imamura et al. 1994). Furthermore, stimulation of H_3-receptors attenuates the noradrenaline overflow during reperfusion following global ischemia in isolated guinea pig hearts by reducing NHE activity, indicating that H_3-receptors modulate nonexocytotic carrier-mediated noradrenaline release during protracted myocardial ischemia (Imamura et al. 1996). In the result, activation of H_3-receptors reduces the incidence of potentially fetal arrhythmias and mitigates the dysfunctional consequence of prolonged myocardial ischemia (Imamura et al. 1996; Levi and Smith 2000). The relevance of H_3-receptors as a major cardioprotective mechanism in myocardial ischemia has been underscored by using mice lacking H_3-receptors. The hearts from H_3-receptor knockout mice have been found to release more than twice as much noradrenaline when subjected to simulated ischemia than those from wild-type mice (Koyama et al. 2003a). Moreover, H_3-receptor knockout mice exhibit increased severity of reperfusion arrhythmias after the hearts are subjected to ischemia (Koyama et al. 2003b).

The exocytotic release of noradrenaline from postganglionic sympathetic neurons requires calcium entry through voltage-dependent calcium channels (Bennett et al. 1998). When the human neuroblastoma cell line stably transfected with the cDNA for the H_3-receptor has been used as a model to study mechanisms of neurotransmitter release, it has been shown that stimulation of H_3-receptors

reduces both the rise in intracellular calcium concentrations and noradrenaline exocytosis in response to membrane depolarization (Silver et al. 2002). Since the H_3-receptor-induced attenuation of noradrenaline exocytosis involves $G\alpha_i$-mediated inhibition of adenylate cyclase leading to decreased cyclic AMP (cAMP) formation, reduced protein kinase A (PKA) activity could result in decreased phosphorylation of voltage-dependent calcium channels, which would be reflected in a decrease in intracellular calcium concentrations (Seyedi et al. 2005) (Fig. 1). In addition to adenylate cyclase inhibition, the H_3-receptor-

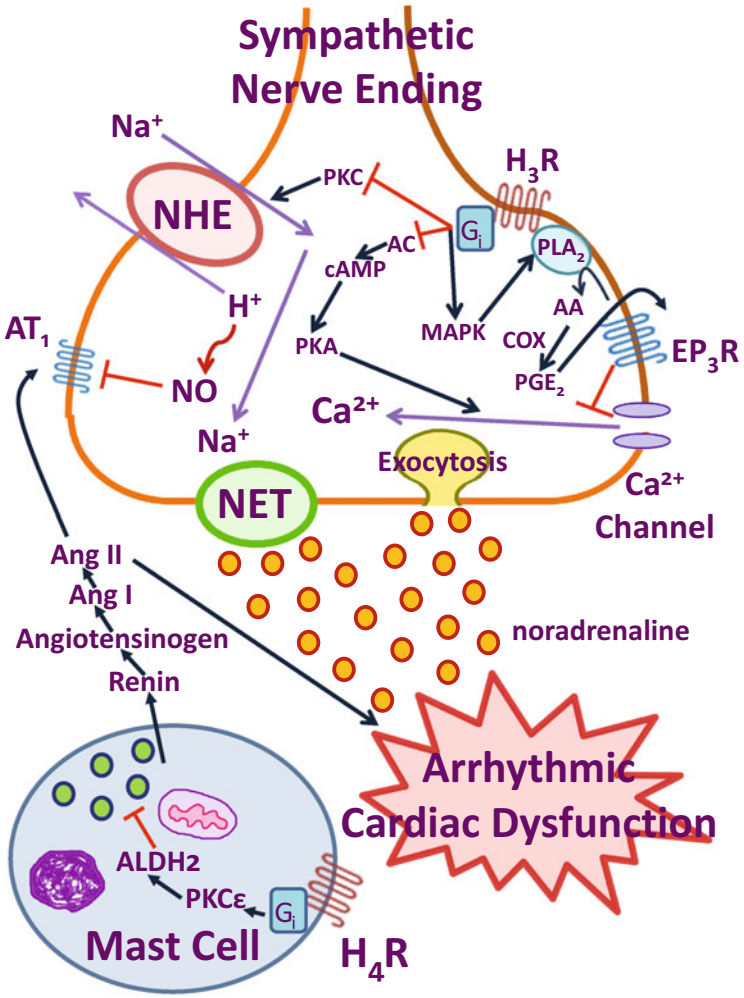

Fig. 1 Scheme illustrating the H_3-receptor (H_3R)- and H_4-receptor (H_4R)-mediated inhibitions of the events that trigger noradrenaline release from sympathetic nerve terminals, mast cell renin release, and, consequently, the development of arrhythmic cardiac dysfunction in myocardial ischemia. See text for details

mediated attenuation of noradrenaline exocytosis is likely to result from the intraneuronal activation of the mitogen-activated protein kinase (MAPK) cascade (Levi et al. 2007). Activated MAPK phosphorylates cytosolic phospholipase A_2, which is then translocated to the cellular membrane, with the consequent formation of arachidonic acid from membrane phospholipids and the subsequent production of PGE_2 via cyclooxygenase. PGE_2 then activates EP_3 receptors on the neuronal membrane, and the $G\beta\gamma_i$ subunit of the EP_3 receptor inhibits calcium entry, thus attenuating noradrenaline exocytosis. Conceivably, the $G\beta\gamma_i$ subunit of the H_3-receptor could also contribute to direct inhibition of calcium entry without MAPK activation. Collectively, the two signaling pathways, impairment of adenylate cyclase-cAMP-PKA function and activation of the MAPK cascade entailing formation of an arachidonate metabolite with anti-exocytotic characteristics, PGE_2, appear to be involved in the H_3-receptor-mediated inhibition of noradrenaline exocytosis from cardiac sympathetic nerve terminals (Fig. 1).

Although it is not fully understood how H_3-receptor stimulation inhibits carrier-mediated release of noradrenaline in protracted myocardial ischemia, carrier-mediated noradrenaline release is associated with neuronal NHE activity, and H_3-receptor activation inhibits NHE by diminishing activity of protein kinase C (PKC) which is known to stimulate NHE (Imamura et al. 1996; Levi and Smith 2000; Hashikawa-Hobara et al. 2012) (Fig. 1). Angiotensin II, formed locally by mast cell-derived renin in myocardial ischemia, is a major NHE activator via AT_1 receptors (Reid et al. 2004, 2007). Activation of AT_1 receptors with angiotensin II elicits reperfusion arrhythmias by a direct action as well as by facilitating noradrenaline release (Maruyama et al. 1999). Then, activation of H_3-receptors may serve a protective function in opposing the noradrenaline-releasing proarrhythmogenic actions of angiotensin II. Indeed, activation of neuronal H_3-receptors opposes the deleterious effects of locally formed angiotensin II, not only by inhibiting NHE but also by reducing expression of AT_1 receptors (Hashikawa-Hobara et al. 2012). Binding to H_3-receptors of histamine, released from local mast cells by the actions of reactive oxygen species produced during myocardial ischemia/reperfusion (Koda et al. 2010), reduces PKC activity. This, in turn, decreases NHE activity (Karmazyn et al. 1999), so that H^+ ions accumulate intraneuronally. Reduced NHE activity sequentially causes intracellular acidification, which stimulates the production of nitric oxide (NO) synthesis. NO has been shown to suppress AT_1 receptor expression (Ichiki et al. 1998). Hence, the H_3-receptor-induced decrease in NHE activity leading to an increased NO synthesis may be responsible for the ultimate decrease in AT_1 receptor expression (Fig. 1). This phenomenon broadens the cardioprotective effects of H_3-receptor activation in myocardial ischemia.

Cardiac mast cells are a critical source of renin (Silver et al. 2004). Indeed, angiotensinogen and angiotensin-converting enzyme are present in cardiac interstitial fluid in concentrations sufficient to ultimately produce angiotensin II (Dell'Italia et al. 1997; Dostel and Baker 1999; Bader et al. 2001), which then act on AT_1 receptors on sympathetic nerve endings, promoting excessive noradrenaline release, thus causing severe arrhythmic dysfunction in cases such as myocardial ischemia (Mackins et al. 2006). Ischemic preconditioning inhibits renin release

from cardiac mast cells and consequent activation of the local renin-angiotensin system (RAS), thereby leading to alleviation of cardiac dysfunction in ischemia/reperfusion. This is associated with the signaling cascade initiated by adenosine, which triggers PKCε-mediated activation of mitochondrial aldehyde dehydrogenase type-2 (ALDH2) in cardiac mast cells (Koda et al. 2010). Activated ALDH2 are known to eliminate toxic aldehydes that accumulate in the ischemic heart (Chen et al. 2008) and to degranulate mast cells (Koivisto et al. 1999; Kawano et al. 2004), thus preventing renin release and its dysfunctional consequences in myocardial ischemia (Koda et al. 2010). H_4-receptors are expressed by hematopoietic cells, including mast cells (Liu et al. 2001b; Zhu et al. 2001). It has been found that stimulation of mast cell H_4-receptors with 4-methylhistamine mimics the cardioprotective anti-RAS effects of ischemic preconditioning which depend on the sequential activation of PKCε and ALDH2 in cardiac mast cells (Aldi et al. 2014). Such effects of 4-methylhistamine could be prevented by selective blockade of H_4-receptors with compound A943931. These findings show that activation of H_4-receptors on the mast cell membrane during ischemia/reperfusion affords cardioprotective anti-RAS effects, which include reductions in renin and noradrenaline release and alleviation of reperfusion arrhythmias (Fig. 1). Interestingly, ischemic preconditioning-mediated anti-RAS cardioprotection is lacking in H_4-receptor knockout mice (Aldi et al. 2014), suggesting that H_4-receptors in cardiac mast cells may be indispensable for the anti-RAS effects of ischemic preconditioning. It is noteworthy that H_4-receptors could also grant cardioprotection by additional mechanisms. Thus, H_4-receptors are present in cardiac sympathetic nerve endings where, similar to H_3-receptors, H_4-receptors inhibit noradrenaline release (Chan et al. 2012). Lastly, H_4-receptor-mediated protected mechanisms seen in the heart may have a significant impact on other organs that have renin-containing mast cells, can suffer from ischemic episodes, and have been shown to be protected by ischemic preconditioning, such as the brain, liver, and kidney.

4 Vascular Effects of Histamine and Its Mechanisms

In most mammalian species, including humans, intravenously administered histamine results in a fall in arterial blood pressure and systemic vasculature resistance, which is mediated by both H_1- and H_2-receptors. However, the effects of histamine on specific regional vascular beds are quite variable. Histamine can produce either vasoconstriction, vasodilatation, or a combination of these responses, depending on the applied dose, administration route, animal species, anatomic location, caliber and tone of the vessel, and distribution of histamine receptor subtypes (Levi et al. 1991).

In general, H_1- and H_2-receptors are present in the smooth muscle of blood vessels, and their stimulation can cause vasoconstriction and vasodilatation, respectively (Marshall 1984). H_2-receptors interact with G_s to activate adenylyl cyclase, whereas H_1-receptors couple to $G_{q/11}$ stimulating PLC. PLC is a key enzyme

responsible for the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate to two intracellular transmitters – 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. 1,2-Diacylglycerol stimulates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP_3) causes mobilization of calcium from intracellular store sites (Rhee 2001; Horowitz et al. 2005). H_1-receptor-induced vasoconstriction has been shown to involve phosphorylation of a specific inhibitor protein for myosin phosphatase by PKC α and δ isoforms (Eto et al. 2001). Thus, PKCα/δ can phosphorylate PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa (CPI-17), which is highly expressed in vascular smooth muscle (Woodsome et al. 2001), to inhibit myosin phosphatase activity and enhance the apparent calcium sensitivity of myosin phosphorylation. On the other hand, H_2-receptor-induced vascular relaxations appear to involve the cAMP-mediated pathway (Fullerton et al. 1996; Santos-Silva et al. 2009). It is currently considered that four main mechanisms are involved in cAMP-mediated vascular relaxations: (1) decreased intracellular calcium levels that can be achieved due to an increase in calcium uptake by the sarcoplasmic reticulum (SR), an inhibition of calcium release from the SR, a rise in intracellular calcium efflux, and/or a reduction in extracellular calcium influx; (2) hyperpolarization of the smooth muscle cell membrane potential through activation of outward potassium channels, inactivation of sodium channels, and/or inactivation of multiple channels; (3) decreased sensitivity of the contractile machinery by decreasing the calcium sensitivity of 20-kDa myosin light chain phosphorylation due to reduced myosin light chain kinase activity and/or increased myosin light chain phosphatase; and (4) reduced sensitivity of the contractile machinery by uncoupling contraction from 20-kDa myosin light chain phosphorylation via a thin-filament regulatory process (Morgado et al. 2012). The cAMP-mediated decrease in intracellular calcium levels may be achieved by (1) decreased release from the SR, via phosphorylation of the SR IP_3 receptor and/or inhibition of IP_3 synthesis; (2) increased sequestration into the SR, via phospholamban phosphorylation and activation of SR Ca^{2+}-ATPase; (3) decreased influx of extracellular calcium; via L-type calcium channels; and (4) increased efflux of intracellular calcium, via stimulation of plasma membrane Ca^{2+}-ATPase (Morgado et al. 2012).

According to the early reports of Van de Voorde and Leusen (1983) and Sato and Inui (1984), histamine-induced relaxations are mediated exclusively by H_1-receptors in the rat thoracic aorta and guinea pig pulmonary artery, respectively. These relaxations are dependent on the presence of the endothelium. Subsequent studies have shown H_1-mediated endothelium-dependent relaxations in different types of blood vessels, including guinea pig aorta (Hide et al. 1988), rat pulmonary artery (Szarek et al. 1992), and monkey coronary artery (Toda 1986). By occupying H_1-receptors on endothelial cells, histamine increases cytosolic calcium concentrations and activates endothelial nitric oxide synthase (eNOS) (Lantoine et al. 1998), which oxidizes its substrate L-arginine to L-citrulline and nitric oxide (NO). The NO produced by eNOS dilates all kinds of blood vessels (Förstermann and Sessa 2012). Stimulation of H_1 receptors with histamine has also been documented to upregulate gene expression of eNOS in human vascular endothelial

cells by a signaling pathway involving calcium/calmodulin-dependent protein kinase II (Li et al. 2003). In addition to stimulation of H_1-receptors, histamine can induce NO release from endothelial cells mediated by H_2-receptors (Kishi et al. 1998). This appears to be associated with increased cAMP content in endothelial cells (Hekimian et al. 1992).

Collectively, although accumulating evidence suggests highly regional and species heterogeneity in histamine receptor subtypes mediating the histamine-induced regulation of vascular tone, histamine appears to produce vascular relaxations mediated by H_2-receptors on smooth muscle cells through the G_s-linked cAMP pathway, while histamine-induced vascular contractions may be mediated by H_1-receptors on smooth muscle cells through the $G_{q/11}$-mediated cellular signaling pathway. Both H_1- and H_2-receptors may also be present on vascular endothelium, and their activation would produce NO production through the elevation of endothelial cytosolic calcium levels, leading to endothelium-dependent vascular relaxations (Fig. 2).

The presence of H_1-receptors on vascular endothelial cells is responsible for histamine's vascular permeability (Niimi et al. 1992). Histamine-induced vascular leakage is an integral of many highly prevalent diseases. Thus, endothelial

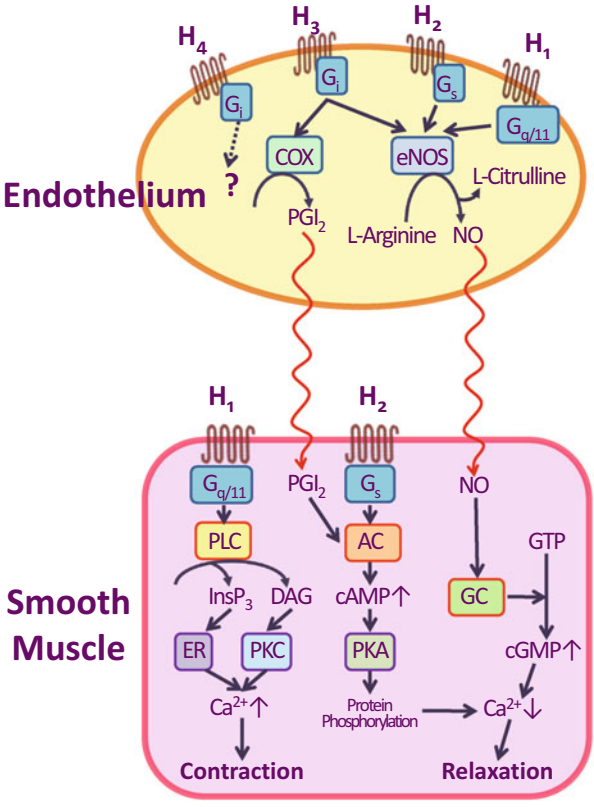

Fig. 2 Schematic presentation of vascular contraction and relaxation mediated by activation of the H_1-receptor (H_1R), H_2-receptor (H_2R), H_3-receptor (H_3R), and H_4-receptor (H_4R), and their possible mechanisms of action. *AC* adenylate cyclase, *GC* guanylate cyclase, *DAG* 1,2-diacylglycerol, *InsP*$_3$, inositol 1,4,5-trisphosphate, *ER* endoplasmic reticulum, *PGI*$_2$, prostacyclin. See text for details

permeability could increase paracellular leakage of plasma fluid and proteins to surrounding tissues and intravazation of tissue-released contents to the blood in the development of multiple diseases related to injury, inflammation, diabetes, and cancer. Recent work suggests that stimulation of H_1-receptors with histamine increases vascular permeability largely by NO-dependent vascular dilation and subsequent increased blood flow and partially by PKC/Rho-associated protein kinase/NO-dependent endothelial barrier disruption (Ashina et al. 2015). What is more, since the work of Zauberman et al. (1969) showing that histamine could induce new blood vessel formation when introduced into the rabbit cornea, many reports have implicated histamine in pathologic angiogenesis (Sörbo et al. 1994; Norrby 1995, 2002), and mechanistic studies have shown this action to be indirect through upregulation of vascular endothelial growth factor-A (VEGF-A) expression (Ghosh et al. 2002). Endogenous mast cell histamine is likely to induce angiogenesis through activation of both H_1- and H_2-receptors (Sörbo et al. 1994). On the other hand, a topical injection of histamine or the H_2-receptor agonist dimaprit can rescue the deficiencies in angiogenesis and granulated tissue formation in HDC knockout mice (Ghosh et al. 2002). Moreover, H_2-receptor antagonists, cimetidine and roxatidine, inhibit angiogenesis and suppress growth of colon cancer implants in syngeneic mice (Tomita et al. 2003; Natori et al. 2005). These findings suggest that the antitumor activity of H_2-receptor antagonists may execute their functions in part by inhibiting tumor-associated angiogenesis. As an added bonus, it has been found that histamine can synergistically enhance basic fibroblast growth factor (bFGF)-induced angiogenesis, which is linked to overproduction of VEGF in endothelial cells through activating H_1- rather than H_2-receptors (Lu et al. 2013).

5 Existence of Postsynaptic H_3- and H_4-Receptors in Vascular Tissues

The vasodilatory effects of H_3-receptor selective agonists were first reported by Ishikawa and Sperelakis (1987) using guinea pig mesenteric artery. This was attributed to their inhibition of sympathetic neurotransmission in adrenergic postganglionic fibers innervating resistant vessels. Subsequently, inhibition by H_3-receptor stimulation with R-α-methylhistamine of vasopressor response to exogenous electrical stimulation of perivascular sympathetic nerves has been found in anesthetized pithed and spontaneously hypertensive rats (Malinowska and Schlicker 1991). Thereafter, vascular presynaptic H_3-receptors have been identified in human saphenous the vein (Molderings et al. 1992), guinea pig pulmonary artery (Rizzo et al. 1995), and anesthetized guinea pigs (Hey et al. 1992). On the other hand, it has been demonstrated that H_3-receptor selective agonists cause endothelium-dependent relaxations in rabbit cerebral artery (Ea Kim et al. 1992) and rat mesenteric artery (Sun et al. 2010), indicating that activation of endothelial H_3-receptors produces vasodilator effects by releasing NO and prostacyclin from endothelial cells (Fig. 2). Furthermore, the presence of H_3-receptor mediating vasodilation which is associated with the release of NO has been shown

in the mesenteric and hindlimb vascular beds of the cat (Champion and Kadowitz 1997, 1998). The application of reverse transcription-PCR and sequencing has demonstrated that rat brain vascular endothelial cells express H_3-receptors (Karlstedt et al. 2013). Very intriguingly, they have also identified the presence of H_4-receptor mRNA in rat brain endothelial cells (Karlstedt et al. 2013). They have revealed that histamine and immepip (an H_3- and H_4-receptor agonist) can activate the ERK1/2 MAPK pathway both in cultured brain microvessel endothelial cells and in vivo in brain blood vessels. The H_4-receptor-specific inverse agonist/antagonist JNJ7777120, but not the H_3-receptor-specific inverse agonist/antagonist ciproxifan, blocks the immepip-induced effect in a dose-dependent manner, indicating that the activation of the ERK1/2 cascade is predominantly conveyed through H_4-receptors (Karlstedt et al. 2013). However, the functional role of endothelial H_4-receptors is still poorly understood, although it can be assumed that H_4-receptors on brain microvessel endothelial cells might affect vascular permeability in the brain.

H_4-receptors are significantly expressed on the epithelium of the gastrointestinal tract, and H_4-receptor expression has been shown to be reduced in some gastric carcinoma samples, especially in advanced cases (Zhang et al. 2012). Furthermore, H_4-receptors are expressed in human breast cancer cells, and H_4-receptor agonists could reduce tumor growth and intratumoral vessels in the human cancer xenograft model of immunodeficient mice (Martinel Lamas et al. 2013), although their immunohistochemical analysis using an H_4-receptor antibody has to be interpreted with utmost caution due to its questionable specificity (Neumann et al. 2012; Seifert 2014). Since tumorigenesis is strongly associated with angiogenesis, these studies suggest that H_4-receptors may negatively regulate angiogenesis. However, the stimulatory role of H_4-receptors in ocular angiogenesis has been observed (Kaneko et al. 2014). H_4-receptors appear to be expressed in macrophages that accumulate around the choroidal neovascularization tissues. Intravitreal injection of JNJ7777120 can suppress laser-induced choroidal neovascularization volume and pathological vessel leakage in mice. Additionally, laser choroidal neovascularization volume is reduced in H_4-receptor knockout mice. It has thus been proposed that H_4-receptors may have a potential as a therapeutic target for choroidal neovascularization in age-related macular degeneration. Taken together, H_4-receptors may play different regulatory roles in angiogenic signaling mechanisms between nonmalignant and malignant tissues, but delineating the exact role of H_4-receptors in vascularization awaits further study.

6 Conclusions

The data reviewed in this chapter establish a role for histamine in cardiovascular pathophysiology. Thus, histamine can elicit a variety of responses in most parts of the cardiovascular system, and the involvements of H_1- and H_2-receptors in many of these responses have been identified. Besides the recognized H_1- and H_2-receptor-mediated cardiovascular responses, it has been shown that presynaptic

and postsynaptic H_3-receptors have a significant impact on cardiovascular variables, such as heart rhythm and vascular tone, under physiological and pathological conditions. However, evidence in support of possible roles of these H_3-receptors in the cardiovascular function or dysfunction is still being gathered. The recently identified histamine receptor H_4-receptor, emerging as a player in both innate and adaptive immune responses, has been indicated to have a potentially critical role in myocardial ischemia-reperfusion arrhythmia, vascular permeability, and vascularization, but its exact functional features have yet to be fully understood. The likely importance of H_3-receptors and H_4-receptors in the pharmacological control of the circulation or in the mediation of cardiovascular events in some pathological states raises an intriguing possibility. Genetic knockout and pharmacological manipulations of H_3- and H_4-receptors as tools to clarify their roles in the cardiovascular system are now available and substantial progress in this area can be anticipated with great interest.

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Changes in Histidine Decarboxylase, Histamine *N*-Methyltransferase and Histamine Receptors in Neuropsychiatric Disorders

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Abstract

Compared to other monoamine neurotransmitters, information on the association between the histaminergic system and neuropsychiatric disorders is scarce, resulting in a lack of histamine-related treatment for these disorders. The current chapter tries to combine information obtained from genetic studies, neuroimaging, post-mortem human brain studies and cerebrospinal fluid measurements with data from recent clinical trials on histamine receptor agonists and antagonists, with a view to determining the possible role of the histaminergic system in neuropsychiatric disorders and to pave the way for novel histamine-related therapeutic strategies.

Keywords

Histamine • Histidine decarboxylase • Histamine receptors • Histamine *N*-methyltransferase • Neurodegenerative diseases • Mood disorders • Intellectual disability

Abbreviations

AD	Alzheimer's disease
CSF	Cerebrospinal fluid
$H_{1–4}R$	Histamine 1–4 receptors
HDC	L-Histidine decarboxylase
HMT	Histamine *N*-methyltransferase
mRNA	Messenger RNA
PD	Parkinson's disease
t-MeHA	Tele-Methylhistamine
TMN	Tuberomamillary nucleus

1 Introduction

In human genes, polymorphisms of monoamine-related neurotransmitter pathways, such as in the serotonin transporter genes, are highly associated with depression and anxiety disorders (Caspi et al. 2003; Homberg and van den Hove 2012; Shan et al. 2014). In addition, the dopaminergic neurons in the substantia nigra tend to be largely lost in Parkinson's disease (PD) (Hirsch et al. 1988). Effective treatments have been developed based upon these monoamine-related changes. For instance, selective serotonin reuptake inhibitors are widely prescribed for the treatment of

depression and anxiety-related disorders, and L-dopa was the first-line treatment for minimizing the motor symptoms of PD. Such pathophysiological relationships between monoamine and neuropsychiatric disorders are as yet unknown for the histamine neurotransmitter system, although fundamental studies have shown that the neuronal histaminergic system is involved in a number of physiological functions, such as the sleep-wake cycle, energy and endocrine homeostasis, sensory and motor functions, cognition and attention (Haas and Panula 2003; Haas et al. 2008; Panula and Nuutinen 2013; Shan et al. 2013b), which are all severely affected in neuropsychiatric disorders.

Recently a series of crucial data were obtained, demonstrating that the key enzyme for the production of neuronal histamine, histidine decarboxylase (HDC) was the cause of a rare familial case of Tourette syndrome (Ercan-Sencicek et al. 2010; Castellan Baldan et al. 2014) (details are reviewed in Pittenger 2017). In the light of the increasing interest in this topic, the time has come to integrate the scattered information on the pathophysiology of the histamine system in order to pave the way for novel therapeutic strategies. In this chapter, we bring together genetic association studies, neuroimaging reports, post-mortem human brain data, cerebral spinal fluid (CSF) measurement and the results of recent clinical trials to discuss the possible association of histamine receptors and key enzymes for histamine synthesis and metabolism with neuropsychiatric disorders.

2 Histamine Synthesis, Metabolism and Receptors in the Brain (Fig. 1)

Neuronal histamine is synthesised by HDC from the amino acid L-histidine, which is exclusively expressed in the tuberomamillary nucleus (TMN) (Fig. 2) of the mammalian brain (Panula and Nuutinen 2013). The enzyme histamine *N*-methyltransferase (HMT) inactivates histamine by transferring a methyl group from *S*-adenosyl-L-methionine to histamine. This is the only known pathway for the termination of histamine neurotransmission in the mammalian central nervous system. Histamine is known to have four types of receptors, all of which are G protein-coupled receptors. Histamine receptors 1–3 ($H_{1-3}R$) are functionally widely expressed in the brain. As several recent authoritative reviews (Passani and Blandina 2011; Schneider et al. 2014a, b; Panula et al. 2015) (for details see Shiroshi and Kobayashi 2017; Monczor et al. 2017; Schlicker and Kathmann 2017; Neumann 2017) have recently discussed the pharmacology, signal pathways and physiological function of histamine receptors we are not discussing these here. Recently accumulated evidence indicates that there is a new G protein-coupled histamine receptor, H_4R, which may also be functionally expressed in the brain (Connelly et al. 2009; Galeotti et al. 2013; Karlstedt et al. 2013). However, due to the controversial opinions regarding the lack of specificity of commercialized antibodies against H_4R (Beermann et al. 2012; Schneider and Seifert 2016) and inability of a H_4R agonist to initiate its downstream signal transduction in the cortex of various species (Feliszek et al. 2015), we will not further discuss this receptor.

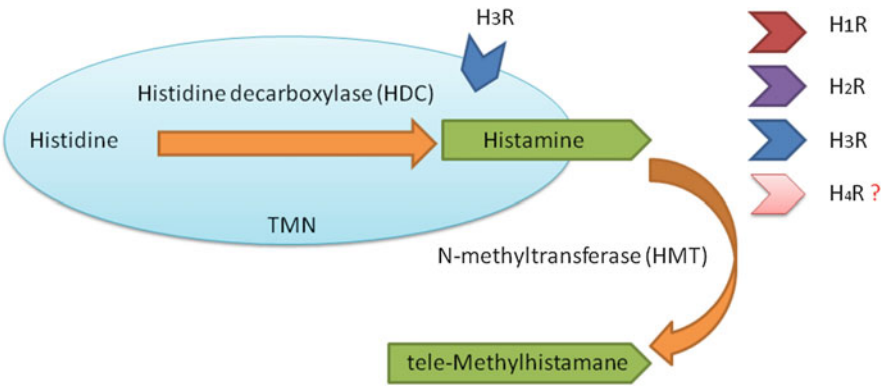

Fig. 1 Schematic illustration of histamine synthesis, metabolism and receptors. Histamine is synthesized by the specific enzyme histidine decarboxylase (HDC) in the tuberomamillary nucleus (TMN). The enzyme histamine *N*-methyltransferase (HMT) inactivates histamine. There are four types of histamine receptors ($H_{1-4}R$). H_3R is also an auto-receptor located pre-synaptically. The functional expression of H_4R in the brain is still unclear, which is indicated by a *question mark*

3 HDC

3.1 HDC Expression and Its Circadian Rhythmicity

Technically, the investigation of HDC is hampered by the fact that HDC-antibodies may also label other monoamine neurons in the substantia nigra, ventral tegmental area and dorsal raphe, by cross-reacting with aromatic L-amino acid decarboxylase (Mizuguchi et al. 1990). Therefore, we opted for in situ hybridization of HDC-messenger RNA (mRNA) for our studies. It should be noted, however, that the expression level of HDC-mRNA is low-to-moderate in post-mortem brain tissues (Liu et al. 2010). Consequently, appropriate specificity tests for both in situ probes and HDC-antibodies are always needed.

Circadian fluctuations of HDC-mRNA expression in the TMN have been reported, both in human (Shan et al. 2012c) and in rodent (Yu et al. 2014). In a group of neurodegenerative disorders, including AD, PD, preclinical PD and Huntington's disease, we observed a loss of this diurnal HDC-mRNA fluctuation (Shan et al. 2012c). These diseases showed symptoms of sleep-wake disturbance, which may, at least partly, be caused by alterations in the arousal-related TMN [reviewed in Lin (2000) and Shan et al. (2015b)]. It is therefore of interest to note that the circadian rhythm of HDC-mRNA expression and brain histamine levels were disturbed in mice that had knockdown of *BMAL1*, a key clock gene in the TMN neurons. These mice also showed functionally altered sleep architecture (Yu et al. 2014).

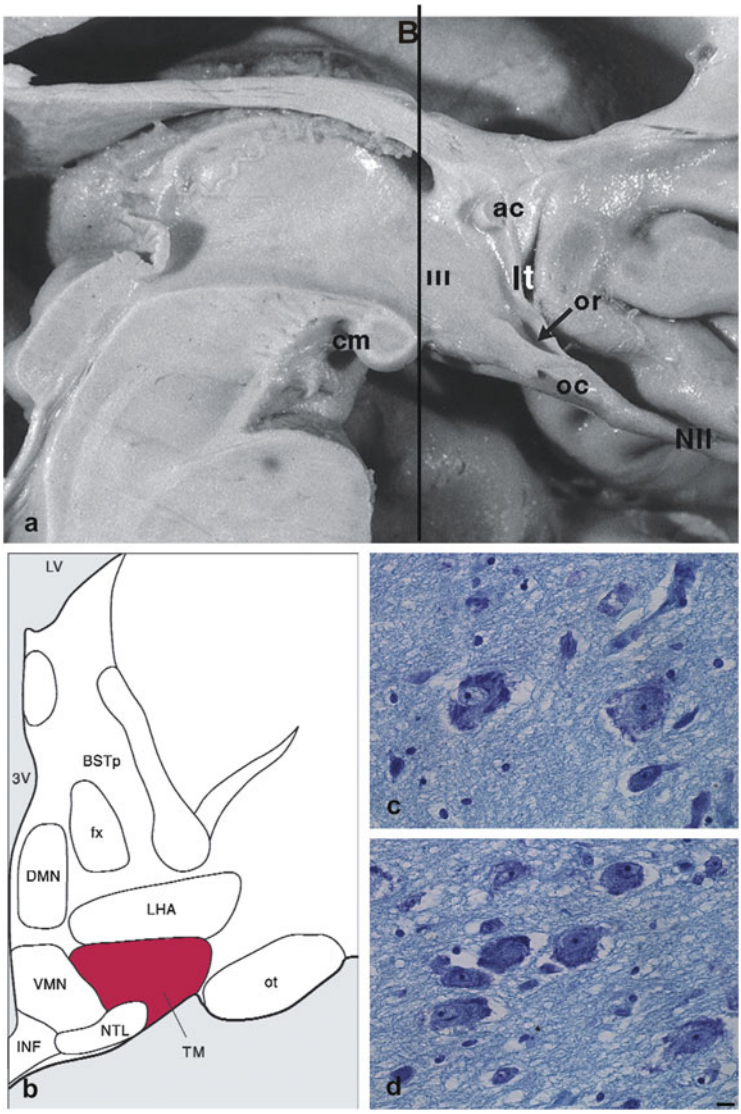

Fig. 2 The neuroanatomy of the tuberomamillary nucleus. (**a**) Medial surface of the human hypothalamus. Line *B* indicating the layer for figure (**b**). *Abbreviations*: *ac* anterior commissure, *cm* corpus mamillare, *lt* lamina terminalis, *NII* optic nerve, *oc* optic chiasm, *or* optic recess, *III* third ventricle. (**b**) The human hypothalamus in representative coronal cuts with the tuberomamillary nucleus highlighted (adapted from Fernandez-Guasti et al. 2000; Fig. 2). *Abbreviations*: *BSTp* bed nucleus of the stria terminalis posterior, *DMN* the dorsomedial hypothalamic nucleus, *OT* optic tract, *Ox* optic chiasma, *fx* fornix, *INF* infundibular nucleus, *LHA* lateral hypothalamus, *LV* lateral ventricle, *NTL* lateral tuberal nucleus, *TM* tuberomamillary nucleus, *VMN* ventromedial hypothalamic nucleus, *3V* third ventricle. (**c**, **d**) Examples of Nissl staining of TM nucleus neurons with typical neuron profiles, scale bar = 5 μm

3.2 Unaltered HDC Expression in Both PD and AD

During the preclinical and clinical PD stages, the HDC mRNA levels were fairly stable, indicating that neuronal histamine production remains intact (Shan et al. 2012d). The total number of histaminergic neurons (Nakamura et al. 1996) and the enzymatic activity of HDC (Garbarg et al. 1983) were also found to be stable in PD patients. The stability is further supported by the unaltered cerebrospinal fluid (CSF) level of the main metabolite of histamine, tele-Methylhistamane (t-MeHA), in PD patients (Prell et al. 1991). We have also observed that, in AD patients, despite the significant loss of histaminergic neurons, the TMN function may be largely compensated by the enhanced histamine production by the remaining histamine neurons, as indicated by the, largely, unaltered HDC-mRNA expression in the TMN (Shan et al. 2012b). The unchanged t-MeHA levels in the CSF of AD patients support this possibility (Motawaj et al. 2010).

3.3 Strong Increase in HDC Immuno-Reactivity in Narcoleptic Patients with Cataplexy: Is It Related to Hallucinations?

The significant loss of hypocretin (orexin) neurons in the hypothalamus is the major cause of narcolepsy with cataplexy (Peyron et al. 2000; Thannickal et al. 2000), which is characterized by clinical symptoms such as excessive daytime sleepiness, hypnagogic/hypnopompic hallucinations, sleep paralysis and disturbed nocturnal sleep (Overeem et al. 2001). Hypnagogic hallucinations occur during the transition from wakefulness to sleep, and hypnopompic hallucinations during the transition between sleep and consciousness.

Some clinical observations have shown that up to 65% of patients suffering from this disorder experienced hallucinations (Fortuyn et al. 2009; Leu-Semenescu et al. 2011). In fact, the symptoms of hypnagogic/hypnopompic hallucinations are so intense in some narcoleptic patients that they may lead to the misdiagnosis of schizophrenia (Douglass et al. 1991, 1993; Howland 1997; Talih 2011). This may also explain that comorbidity of narcolepsy and schizophrenia was often reported (Canellas et al. 2014; Chen et al. 2014; Plazzi et al. 2015). Narcoleptic animal models are generally generated based exclusively upon disturbed hypocretin (orexin) pathways. The major clinical symptoms can be found in these animal models, such as a short onset of rapid eye movement, cataplexy and fragmented sleep during the sleep stages (Chemelli et al. 1999; Hara et al. 2001; Tabuchi et al. 2014; Shan et al. 2015a). However, there is no way of telling whether these animals may have hallucinations. In 2013, two research groups independently observed that HDC immuno-reactivity is greatly increased in the TMN of narcoleptic patients (John et al. 2013; Valko et al. 2013), which indicates that not only the hypocretin (orexin) system, but also other systems, such as the histaminergic system, may be involved in narcolepsy. It should be noted that none of the narcoleptic animal models showed this HDC-neuropathology (John et al. 2013). It may be speculated that the strong increase in the number of histamine neurons may, at least partly, contribute to hallucinations found in narcolepsy. This possibility is supported by the

observation that patients with Huntington's disease, a disease that is reported to be accompanied by schizophrenia-like symptoms such as delusions and hallucinations (Tsuang et al. 1998, 2000; Correa et al. 2006), also had a significantly increased histamine production in the TMN (van Wamelen et al. 2011).

4 Histamine *N*-Methyltransferase (HMT)

4.1 HMT Mutations and Intellectual Disability

Recently, two homozygous *HMT* mutations (i.e. p.Gly60Asp and p.Leu208Pro) were identified in patients suffering from non-syndromic autosomal recessive intellectual disability in two unrelated consanguineous families of Turkish and Kurdish ancestry (Heidari et al. 2015). The patients from both families did not present with congenital malformations, facial dysmorphisms, neurological abnormalities or autistic features.

Subsequently, an in vitro study showed that, although the p.Gly60Asp mutation does not affect HMT expression at the mRNA or protein level, the enzymatic activity of HMT, the thermal stability and the affinity of binding to *S*-adenosyl-L-methionine were disrupted by a p.Gly60Asp mutation (Heidari et al. 2015). The p.Leu208Pro mutation was found to result in misfolding and rapid degradation of HMT protein (Heidari et al. 2015). Subsequent molecular dynamic simulations showed that the p.Leu208Pro mutation perturbs the helical character and disrupts the interaction with the adjacent β-strand, which is involved in the binding and correct positioning of histamine (Tongsook et al. 2016). This novel finding calls for detailed behaviour characterization of HMT knockout animals.

4.2 HMT in PD

Animal experiments have shown that increased histamine levels in the substantia nigra may cause a degeneration of dopaminergic neurons (Vizuete et al. 2000; Liu et al. 2007). HMT, the brain's main degradation enzyme for histamine, may thus play an important role in the pathogenesis of PD, but human studies do not support such a relationship.

A polymorphism of the *HMT* gene, rs11558538, causes the amino acid substitution Thr105Ile and leads to the formation of misfolded HMT protein, which is cleared by proteasomes, and therefore to a decreased HMT enzymatic activity (Pang et al. 2001). Individuals who are heterozygous for the 105Ile allele have 30–50% lower HMT activity, while individuals who are homozygous for the 105Ile have decreased enzyme activity of around 60% (Preuss et al. 1998; Horton et al. 2001; Rutherford et al. 2008). Several previous studies have revealed that the lower HMT activity alleles protect against PD development (Agundez et al. 2008; Ledesma et al. 2008; Palada et al. 2012; Yang et al. 2015). A recent meta-analysis, based upon five available studies involving 2,108 patients with PD and 2,158

controls, confirmed that decreased histamine metabolism in the central nervous system could play a role in protecting against PD (Jimenez-Jimenez et al. 2016).

In addition, there are a number of post-mortem studies that do not point to a protective role of HMT against the pathogenesis of PD. A significantly higher concentration of histamine – but not of t-MeHA (Rinne et al. 2002) – and accumulated histaminergic fibres (Anichtchik et al. 2000) was found in the substantia nigra, caudate nucleus and putamen of PD patients. Moreover, we reported an augmented HMT-mRNA expression in the same brain regions in PD patients (Shan et al. 2012a). It is as yet not clear whether the up-regulation of HMT-mRNA is induced by the increased levels of local histamine, but HMT does not appear to play a protective role in the inactivation of histamine, as the levels of t-MeHA remained unaltered (Rinne et al. 2002). Moreover, we also observed a negative correlation between HMT-mRNA expression in the substantia nigra and the disease duration of PD patients (Shan et al. 2012a). This suggested that the more serious (and thus the shorter lasting) the disease, the more HMT-mRNA is expressed. Based upon all these data, one could propose that the process of translation from mRNA to functional enzyme may be impaired in the basal ganglia of PD patients.

4.3 HMT Expression in Cerebral Cortex Related to Cognition and Mood State

As we discussed previously, the functional up-regulation of the histaminergic system in Huntington's patients may be involved in the cognitive impairment of this disease. An up-regulation of HMT-mRNA was also found in the inferior frontal gyrus of Huntington's disease patients (van Wamelen et al. 2011). In addition, increased histamine production as reflected by the HDC-mRNA expression (van Wamelen et al. 2011) and elevated CSF levels of histamine metabolites (Prell and Green 1991) were both reported in Huntington's disease.

Altered metabolic activity in the anterior cingulate cortex (ACC) has been consistently reported in the induction of the depressive state in major depressive disorders, and ACC metabolism and connectivity were found to be reversed by pharmacological treatment (Mayberg et al. 2000) or deep brain stimulation (Mayberg et al. 2005), which successfully improved the symptoms of depression (Kennedy et al. 2011). The lower HMT-mRNA expression in the ACC of depression patients (Shan et al. 2013a) may imply histamine level/turnover alterations in this pivotal brain region. This is in line with a reduction of the H_1R binding in the same brain region (Kano et al. 2004).

5 H_1R

5.1 Modulation of Cognition and Mood

A reduction of H_1R binding was reported in several neuropsychiatric disorders. Positron emission tomography studies showed that H_1R binding, detected by the radioligand for H_1R, ^{11}C-doxepin, was much lower in the frontal cerebral cortex of

depressive patients compared to matched controls (Kano et al. 2004; Yanai and Tashiro 2007). Interestingly, H_1R binding in the frontal cortex and cingulate gyrus decreased in relation to self-rated depressive scale scores (Kano et al. 2004). It was also reported that the amount of H_1R binding is reduced in the frontal and temporal brain areas of AD patients (Higuchi et al. 2000). More importantly, there is a correlation between H_1R binding and severity of cognitive symptoms (Higuchi et al. 2000).This alteration seems to be specifically receptor-dependent, because the binding of another histamine receptor, H_2R, was unchanged in AD prefrontal cortex (Perry et al. 1998). In a post-mortem study, the patients with chronic schizophrenia also showed a significant reduction in H_1R binding in the frontal cortex (Nakai et al. 1991).

Notably, a lack of changes in the H_1R-mRNA was observed in the frontal cortex in depression (Shan et al. 2013a) as well as in AD (Shan et al. 2012b) in our post-mortem studies. The possible deficits in the translation of H_1R-mRNA to the functional H_1R in the cortex in these disorders deserve future attention.

5.2 H_1R Antagonists as a Treatment for Insomnia

Many H_1R antagonists are able to cross the blood–brain barrier and cause drowsiness (Lieberman 2009). Diphenhydramine, chlorpheniramine, doxylamine and brompheniramine are over-the-counter medicines with H_1R antagonistic activity. They have been prescribed to treat allergies, cold symptoms, itching, nausea and insomnia (Krystal et al. 2013). It should be noted that some antidepressants and antipsychotics with a major effect on cholinergic, dopaminergic, serotoninergic and adrenergic receptors may also act on histamine-related mechanisms that show beneficial effects on insomnia (Krystal 2009).

A placebo-controlled trial using the selective H_1R antagonist doxepin in patients with chronic primary insomnia (Roth et al. 2007) showed a major effect in terms of preventing early morning awakening, as well as in terms of improved sleep in the second part of the night.

6 H_2R and Schizophrenia

An early study demonstrated that schizophrenic patients had a higher incidence of the H2R649G allele polymorphisms located in the coding region of the H_2R gene (Orange et al. 1996). However, a follow-up study with a larger sample size did not support this association of the allelic variation with schizophrenia (Ito et al. 2000). In early preliminary open-label clinical trials, the H_2R-antagonist famotidine was shown to have an antipsychotic effect and to reduce negative schizophrenic symptoms (Kaminsky et al. 1990; Oyewumi et al. 1994; Rosse et al. 1996). The antipsychotic effects of famotidine were confirmed in a recent randomized clinical trial. Obvious improvements in both positive and negative symptoms of schizophrenia patients were obtained in that study (Meskanen et al. 2013). The authors of

this study pointed out that famotidine treatment requires high dosage because of its low blood–brain barrier penetration. However, a meta-analysis that pooled eight double-blind randomized placebo-controlled trials with the H_2R-antagonists (famotidine, nizatidine or ranitidine) as adjunctive therapy did not observe any effect on schizophrenic symptoms (Kishi and Iwata 2015).

7 H_3R

7.1 Treatment of Alzheimer's Disease and Schizophrenia

Various ongoing clinical trials study the use of H_3R-antagonist/inverse agonist for the treatment of AD, PD, narcolepsy, schizophrenia and attention-deficit hyperactivity disorder (Brioni et al. 2011; Passani and Blandina 2011). The neurobiological basis of this application is that H_3R-antagonists/inverse agonists stimulate the release of histamine, GABA, acetylcholine and dopamine in the brain (Medhurst et al. 2007; Galici et al. 2009; Giannoni et al. 2010). However, no beneficial effects emerged in terms of improving cognitive functioning in the application of H_3R-antagonists/inverse agonist for the treatment of AD or mild-to-moderate AD patients (Egan et al. 2012, Grove et al. 2014, Kubo et al. 2015). On the other hand, this is in line with the small increase in H_3R-mRNA we observed in female AD patients (Shan et al. 2012b), together with the insignificant changes of H_3R-binding density in the prefrontal cortex reported by another post-mortem study (Medhurst et al. 2007). To date, H_3R inverse agonists also failed to show a therapeutic effect in schizophrenia (Egan et al. 2013, Haig et al. 2014, Jarskog et al. 2015).

7.2 Treatment for Hypersomnia

It is noted, however, that preclinical and clinical data indicate the positive effectiveness of H_3R-antagonist/inverse agonist for the treatment of daytime sleepiness in several neurological disorders associated with hypersomnia (Passani and Blandina 2011). In a narcolepsy animal model, i.e. the hypocretin (orexin)-knockout mice, the administration of Pitolisant yielded significant improvement of the key symptoms of sleepiness, and it decreased direct onsets of rapid eye movement sleep from wakefulness, which is a diagnostic criterion for narcolepsy (Lin et al. 2008). In both adults and children with narcolepsy, Pitolisant ameliorated excessive daytime sleepiness (Lin et al. 2008; Inocente et al. 2012; Dauvilliers et al. 2013). Pitolisant has, therefore, been approved as orphan drug for narcolepsy.

To date, only few published reports document the treatment effects of H_3R-antagonist/inverse agonist on excessive sleepiness in PD, but various clinical trials are still ongoing [according to the clinical trial data base (https://clinicaltrials.gov)].

8 Conclusion and Perspective

Recent data indicate that alterations in several components of the histaminergic system may contribute to the pathogenesis of neuropsychiatric disorders such as narcolepsy, schizophrenia, depression, AD and PD (Table 1). The histaminergic compounds were shown to have novel therapeutic applications. The increased number of histamine neurons (marked by HDC) in the narcoleptic brain is hypothesized to contribute to the hypnagogic/hypnopompic hallucinations of this disorder. HMT was presumed to play a role in the pathogenesis of PD, but the animal data and human genetic, post-mortem studies failed to show a consistent effect. In addition, two rare *HMT* gene mutations were found to lead to intellectual disability. They deserve to be studied in HMT knockout animal model. A reduction of H_1R binding in the cerebral cortex was observed in AD, depression and schizophrenia, which may imply that H_1R availability is associated with cognitive functions and mood states. The H_1R knockout animal seems to provide a great opportunity for further studies of such an involvement in cognition and anxiety. H_1R antagonists are a potential effective treatment for insomnia. Preliminary results have shown that the H_2R-antagonist induced a significant improvement of schizophrenic symptoms. Novel antagonists with higher penetration rate through the blood–brain barrier and follow-ups in clinical trials are urgently needed. One of the H_3R-antagonist/inverse agonists, Pitolisant, has been approved for clinical treatment for narcolepsy. The effectiveness of other H_3R-antagonist/inverse agonist for the treatment of excessive daytime sleepiness has to be studied in animal models and clinical trials. The functional expression of H_4R is not yet clear. However, recently an anxiety and

Table 1 Overview of key alterations of brain histaminergic system in neuropsychiatric disorders

	Histamine production		
Disorders	TMN neurons	HDC-mRNA	Key changes of histamine metabolism and receptors in brain areas
PD	–	–	SN (mRNA HMT ↑; H_3R ↓, HA level ↑; H_3R binding ↑) PU mRNA (HMT ↑; H_3R↓;H_4R ↑;HA level ↑), t-MeHA level in CSF–
AD	↓(−57%)	–/↓(−20%)	PFC mRNA (HMT and H_3R ↑), HA level in brain↑/↓ in CSF–/↑/↓
Huntington's disease	–	↑(+63%)	IFG mRNA (H_1R ↑; H_3R ↑; HMT ↑);CN mRNA (H_2R↓; H_3R↓), H_2R and H_3R binding ↓. H_1R binding ↑, t-MeHA in CSF↑
Depression	–	–	ACC mRNA (HMT↑);H_1R binding by PET scanning ↓in ACC and PFC
Narcolepsy	64 or 94%↑	N.A.	HA level in CSF–/↓, t-MeHA level in CSF–

Notes and Abbreviations: ↑ increase, – unaltered, ↓ decrease, *CSF* cerebrospinal fluid, *CN* Caudate nucleus, *HDC* histidine decarboxylase, *HMT* histamine methyltransferase, *LB*, *LN* Lewy bodies, Lewy neurites, *PU* putamen, *PFC* prefrontal cortex, *IFG* Inferior frontal gyrus, *SN* substantia nigra, *TMN* tuberomamillary nucleus, *NFT* neurofibrillary tangles, *$H_{1–4}R$* histamine-1-4-receptor, *t-MeHA* tele-melthyhistamine

despair behavioural phenotype of a histamine H_4R knockout mice has been identified by the use of a light–dark box and the tail suspension test (Sanna et al. 2017). The possible role of this novel histamine receptor in the central nervous system deserves further research in both animal models and patients with neuropsychiatric disorders.

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Role of the Histamine H_3 Receptor in the Central Nervous System

Eberhard Schlicker and Markus Kathmann

Contents

Abstract

The $G_{i/o}$ protein-coupled histamine H_3 receptor is distributed throughout the central nervous system including areas like cerebral cortex, hippocampus and striatum with the density being highest in the posterior hypothalamus, i.e. the area in which the histaminergic cell bodies are located. In contrast to the other histamine receptor subtypes (H_1, H_2 and H_4), the H_3 receptor is located presynaptically and shows a constitutive activity. In detail, H_3 receptors are involved in the inhibition of histamine release (presynaptic autoreceptor), impulse flow along the histaminergic neurones (somadendritic autoreceptor) and histamine synthesis. Moreover, they occur as inhibitory presynaptic heteroreceptors on serotoninergic, noradrenergic, dopaminergic, glutamatergic, GABAergic and

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perhaps cholinergic neurones. This review shows for four functions of the brain that the H_3 receptor represents a brake against the wake-promoting, anticonvulsant and anorectic effect of histamine (via postsynaptic H_1 receptors) and its procognitive activity (via postsynaptic H_1 and H_2 receptors). Indeed, H_1 agonists and H_3 inverse agonists elicit essentially the same effects, at least in rodents; these effects are opposite in direction to those elicited by brain-penetrating H_1 receptor antagonists in humans. Although the benefit for H_3 inverse agonists for the symptomatic treatment of dementias is inconclusive, several members of this group have shown a marked potential for the treatment of disorders associated with excessive daytime sleepiness. In March 2016, the European Commission granted a marketing authorisation for pitolisant (Wakix^R) (as the first representative of the H_3 inverse agonists) for the treatment of narcolepsy.

Keywords
Epileptic seizures • Food intake • Histamine H_3 receptor • Histaminergic neurones • Learning and memory • Narcolepsy • Noradrenergic neurones • Pitolisant • Presynaptic receptors • Sleep-wake regulation

1 Introduction

The $G_{i/o}$ protein-coupled histamine H_3 receptor was first identified as a functional entity (Arrang et al. 1983; rat) and cloned 16 years later (Lovenberg et al. 1999; human). The gene of the hH_3 receptor is located on chromosome 20q13.33, and the corresponding receptor protein consists of 445 amino acid residues. Subsequent studies (reviewed in Panula et al. 2015) revealed that the hH_3 receptor gene consists of three exons/two introns or four exons/three introns and that multiple receptor isoforms exist (Leurs et al. 2005; Bongers et al. 2007). Genetic polymorphisms have also been described (Hancock et al. 2003). Another typical property of the hH_3 receptor is its ability to form homodimers (Panula and Nuutinen 2013) or heterodimers, e.g. with the dopamine D_1 (Ferrada et al. 2009) and D_2 receptor (Ferrada et al. 2008). The abovementioned properties, which in part also hold true for the H_3 receptor from animals (e.g. mouse, rat and guinea pig), point to a marked biological diversity of this receptor which has so far not been fully deciphered. Only selected aspects of the molecular biology of the H_3 receptor will be covered here (for a more detailed description, see Panula et al. 2015). The H_3 receptor is expressed on autonomic and sensory neurones, endocrine cells and vascular endothelium (for review, see Panula et al. 2015) but mainly in the central nervous system (CNS). This review will be dedicated to the latter aspect only. In detail, we will first discuss basic properties of the H_3 receptor (2.1–2.3) and will then turn to the role played by this receptor in four selected functions of the brain (3.1–3.4).

2 Anatomy, Physiology and Pharmacology of H_3 Receptors

2.1 Anatomy

The H_3 receptor differs from the other three histamine receptor subtypes (H_1, H_2 and H_4) with respect to its presynaptic location. The investigation in which the H_3 receptor was described for the first time (Arrang et al. 1983) and subsequent transmitter release and electrophysiological studies revealed that this receptor occurs on the axon terminals of histaminergic and non-histaminergic neurones. As suggested by electrophysiological work, the H_3 receptor is also located somadendritically on histaminergic neurones (Stevens et al. 2001). With these functional data in mind, one would expect that H_3 receptors can be detected anatomically in the perikarya of the histaminergic neurones and in the areas to which these neurones project. The histaminergic system like other amine transmitter systems (e.g. the serotoninergic and noradrenergic one) shares the property that the perikarya are restricted to a relatively small basal area of the brain from which they diffusely project to many parts of the CNS. In general, binding density (when, e.g. compared to that of cannabinoid CB_1 receptors) is relatively low (Table 1). Autoradiography of the rat brain with ^{3}H-*R*-α-methylhistamine (Pollard et al. 1993) revealed that dense labelling occurs in the perimammillary area of the hypothalamus, i.e. the area of the histaminergic cell bodies (Pollard and Schwartz 1987). Some degree of binding is also detected in the locus coeruleus and the raphe nuclei, i.e. the areas containing the noradrenergic and serotoninergic perikarya. Other areas of the brain including cerebral cortex, striatum and hippocampus show high degrees of labelling. In the cortex, binding decreases from rostral to caudal and is higher in deep than in superficial layers. A low degree of binding is found in most parts of the brainstem, the cerebellum and the spinal cord. A similar pattern of H_3 receptor distribution was obtained when other radioligands were used including ^{3}H-*S*-methylthioperamide (Yanai et al. 1994), ^{125}I-iodoproxyfan (Ligneau et al. 1994), ^{125}I-iodophenpropit (Jansen et al. 1994) and ^{18}F-ST889 (Selivanova et al. 2012). H_3 receptor distribution has also been studied in the brain of humans (Anichtchik et al. 2001; Jin et al. 2002; Jin and Panula 2005; based on ^{3}H-N^{α}-methylhistamine) and mice (Chazot et al. 2001; based on an immunohistochemical analysis), yielding comparable results when compared to the rat brain.

Table 1 Comparison of H_3 and CB_1 receptors with respect to receptor density and stimulation of ^{35}S-GTPγS binding in mouse brain cortex membranes

	H_3 receptor[a]	CB_1 receptor[b]
Receptor density (fmol/mg protein)	111 ± 2	444 ± 52
Maximum increase in ^{35}S-GTPγS binding (% of basal binding)	~20	79 ± 2

Means (± standard error of the mean)

[a]From Nickel et al. (2001). Receptor density was determined with ^{3}H-N^{α}-methylhistamine. *R*-α-Methylhistamine was used to stimulate ^{35}S-GTPγS binding

[b]From Nakazi et al. (2000). Receptor density was determined with ^{3}H-WIN 55,212-2. For stimulation of ^{35}S-GTPγS binding, WIN 55,212-2 was used

The possibility that the tracers also bound to mast cells had to be considered since this cell type accounts for up to 50% of the histamine content in the whole brain and for up to 90% in the thalamus (Hough 1988). It would be an intriguing idea that histamine release not only from histaminergic neurones but also from mast cells is subject to a negative feedback involving H_3 receptors. However, mast cells are not endowed with H_3 receptors (Dimitriadou et al. 1994; Lippert et al. 2004), as opposed to the other three histamine receptor subtypes (Lippert et al. 2004). Nonetheless, an indirect type of negative feedback involving mast cells has been revealed in peripheral tissues (Dimitriadou et al. 1994). Thus, histamine released from mast cells activates H_3 receptors present on neuropeptide-containing neurones and thereby restricts the release of, e.g. calcitonin gene-related peptide (CGRP) which in turn facilitates mast cell degranulation.

After the H_3 receptor had been cloned (Lovenberg et al. 1999), it became possible to compare H_3 receptor binding with H_3 receptor mRNA expression. Such studies have been carried out both for the rat (Pillot et al. 2002) and the human brain (Anichtchik et al. 2001; Jin et al. 2002; Jin and Panula 2005) and are particularly interesting for a receptor which is located presynaptically since its mRNA is located in the perikarya, whereas receptor binding is located on the axon terminals to which the receptor protein is transported along the axons. Some major findings from the comprehensive study by Pillot et al. (2002) in the rat brain will be summarized here. Moderate to high mRNA expression as opposed to low binding has been found in the locus coeruleus and the raphe nuclei and these data are compatible with the view that noradrenergic and serotoninergic neurones are equipped with presynaptic receptors, respectively. The combination of high mRNA expression and low binding is also typical for the pyramidal cells of the CA1 and CA3 fields of the hippocampus and the cerebellar Purkinje cells suggesting that the nerve endings rather than the perikarya of both fibre tracts are endowed with H_3 receptors. A different situation was described for the tuberomammillary nucleus; the very high amount both of binding and mRNA suggests the occurrence of somadendritic H_3 receptors in this brain area and of presynaptic H_3 receptors on the histaminergic axon terminals.

2.2 Physiology

Transduction mechanisms commonly encountered in $G_{i/o}$ protein-coupled receptors, e.g. the CB_1 receptor (Howlett 2005) have also been considered for the H_3 receptor. Thus, inhibition of cAMP accumulation was shown for recombinant (Lovenberg et al. 1999) and native H_3 receptors (Sánchez-Lemus and Arias-Montaño 2004). Moreover, activation of mitogen-activated protein kinase (MAP kinase) was revealed both for recombinant (Drutel et al. 2001) and native H_3 receptors (Giovannini et al. 2003). Blockade of voltage-gated Ca^{2+} channels is shared by native H_3 receptors (Stevens et al. 2001; Lundius et al. 2010). The opening of K^+ channels representing an established transduction process for CB_1 receptors has also been considered for H_3 receptors, but has not been proven yet

(discussed in De Luca et al. 2016). Additional transduction pathways shown for H_3 receptors have been reviewed in Bhowmik et al. (2012) and Panula et al. (2015). Transduction pathways potentially occurring in a presynaptic axon terminal, i.e. on a site which is particularly typical for H_3 receptors (see below), are shown in Fig. 1.

H_3 receptors have at least three functions. First, they serve as presynaptic inhibitory receptors. Several locations have been identified in transmitter release studies (on slices or synaptosomes; e.g. Arrang et al. 1983; Garbarg et al. 1992) and in electrophysiological studies (e.g. Doreulee et al. 2001; Lundius et al. 2010). Figure 2 gives an example how an H_3 receptor was identified in superfused cortical

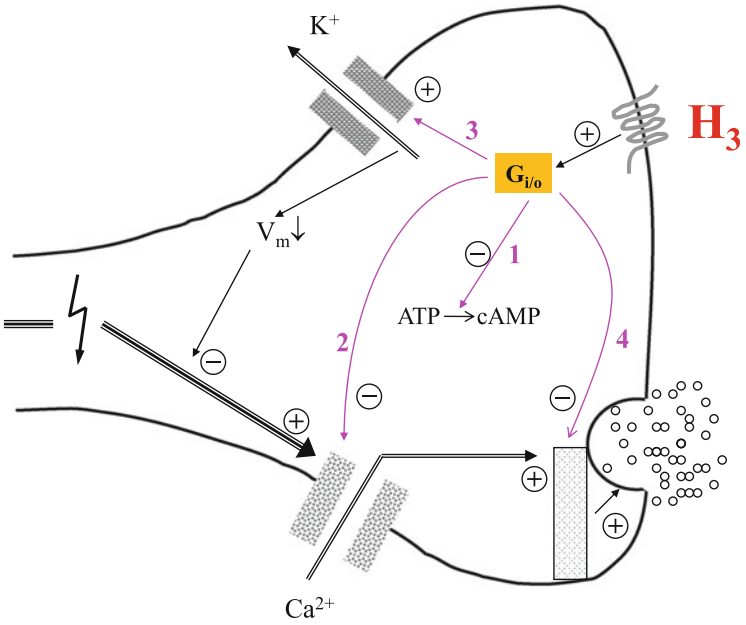

Fig. 1 Transduction pathways potentially occurring in axon terminals of histaminergic and/or non-histaminergic neurones. An action potential (*arrow*) invading the axon terminal leads to an increase in Ca^{2+} influx via voltage-dependent Ca^{2+} channels, activation of the release machinery (*vertical bar*), fusion of vesicles with the synaptic membrane and transmitter release. The H_3 receptor activates $G_{i/o}$ proteins which in turn may be coupled to four transduction pathways. (1) Inhibition of adenylate cyclase is a well-established transduction pathway for the H_3 receptor (Lovenberg et al. 1999; Sánchez-Lemus and Arias-Montaño 2004), but does not play a role in the inhibition of transmitter release (reviewed in Kubista and Boehm 2006). (2) By contrast, the inhibition of voltage-dependent Ca^{2+} channels plays a major role in the H_3 receptor-mediated inhibition of transmitter release (e.g. Brown and Haas 1999; Lundius et al. 2010). (3) Activation of K^+ efflux followed by a decrease of the membrane potential (V_m) and subsequent inhibition of action potential propagation is involved in the inhibitory effect of some cannabinoid CB_1 receptors on transmitter release (reviewed in Schlicker and Kathmann 2001; Szabo and Schlicker 2005), but a role of this mechanism for the H_3 receptor has so far not been established (De Luca et al. 2016). (4) The same holds true for a direct and Ca^{2+}-*in*dependent inhibitory effect on the transmitter release machinery, which although shown for some CB_1 receptors (reviewed in Schlicker and Kathmann 2001; Szabo and Schlicker 2005) does not apply for H_3 receptors (Brown and Haas 1999). The symbols + and – designate a stimulatory and inhibitory influence, respectively

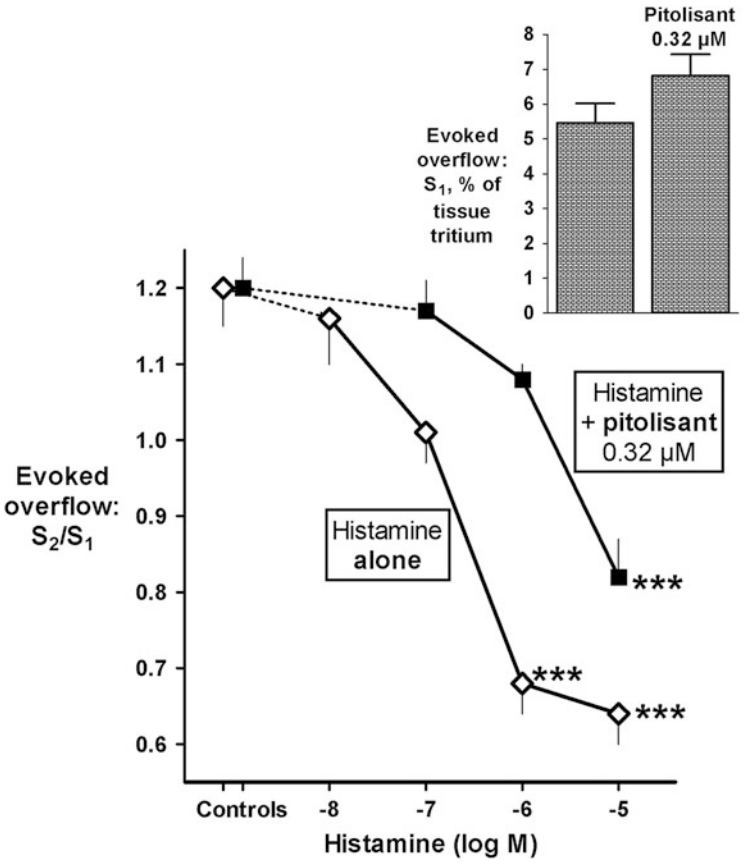

Fig. 2 Effects of histamine and pitolisant (tiprolisant, BF2.649, FUB 649) on the electrically evoked tritium overflow from superfused mouse brain cortex slices preincubated with ^{3}H-noradrenaline. Tritium overflow, which represents quasi-physiological noradrenaline release, was evoked electrically (0.3 Hz) after 40 and 90 min (S_1 and S_2). Histamine was added to the medium before and during S_2 only, whereas pitolisant was present throughout superfusion. To quantify the effect of histamine (studied in the absence or presence of pitolisant), the ratio of the overflow evoked by S_2 over that evoked by S_1 was calculated (S_2/S_1). Pitolisant shifted the concentration-response curve of histamine to the right, yielding an apparent pA_2 value of 7.7. To quantify the effect of pitolisant by itself on the evoked overflow, the tritium overflow given as percent of tissue tritium was determined (*inset*). Since the latter parameter markedly varies from animal to animal, the effect of pitolisant did not reach a significant level (although tritium overflow was higher than the control in each of the 6 experiments). Two alternative explanations for the slight facilitatory effect of pitolisant, i.e. blockade of presynaptic α_2-autoreceptors or the neuronal noradrenaline transporter, can be excluded since the experiments were routinely performed in the presence of blockers of both mechanisms. The diagrams are based on the paper by Liedtke et al. (2003) in which only the pA_2 value of pitolisant had been listed. Means (+ or – standard error of the mean) of 5–6 experiments. ***$P < 0.001$

slices. H_3 receptors occur as presynaptic autoreceptors on the histaminergic axon terminals (Fig. 3; Arrang et al. 1983). However, they are also present on axon terminals of non-histaminergic neurones (heteroreceptors) containing the following transmitters: serotonin, noradrenaline, dopamine, GABA, glutamate and acetylcholine (Fig. 3, which also contains references). Presynaptic H_3 receptors have been studied in various brain areas of animals but were also found in the human cortex (histaminergic neurones – Arrang et al. 1988; noradrenergic neurones – Schlicker et al. 1999). Presynaptic H_3 receptors are inhibitorily coupled to voltage-dependent Ca^{2+} channels (Lundius et al. 2010).

Second, H_3 receptors serve as inhibitory somadendritic receptors (Fig. 3), i.e. they inhibit the firing rate along the histaminergic neurones. This effect, which has been identified in electrophysiological studies, is related to the blockade of Ca^{2+} channels (Stevens et al. 2001). Third, as suggested by studies on cortical

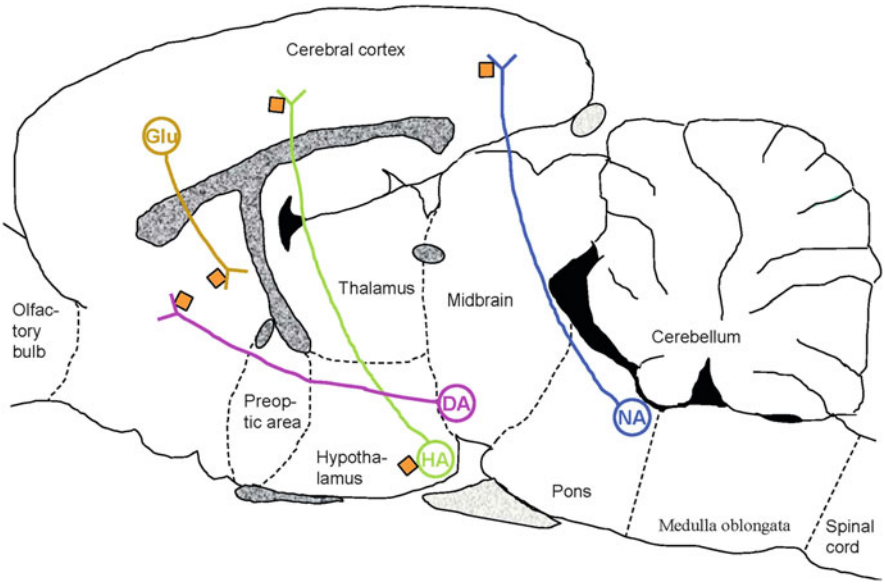

Fig. 3 Presynaptic and somadendritic H_3 receptors (*red squares*) in the rat brain. The picture represents a rat brain cut in a sagittal plane 0.4 mm lateral to the midline since almost all major brain regions can be seen at this level (redrawn from Paxinos and Watson 1997). Schematic drawing of four neurones with perikaryon (*circle*), axon (which may have a course entirely different from that shown here) and axon terminals (V) are shown. The histaminergic (HA) neurone is endowed with presynaptic (Arrang et al. 1983) and somadendritic (Stevens et al. 2001) H_3 autoreceptors. Presynaptic H_3 heteroreceptors occur on serotoninergic (not shown; Schlicker et al. 1988), noradrenergic (NA; Schlicker et al. 1989), dopaminergic (DA; Schlicker et al. 1993), GABAergic (not shown; Garcia et al. 1997) and glutamatergic neurones (Glu; Doreulee et al. 2001). For a comprehensive list of references also including species other than the rat and for a detailed discussion of the controversial issue of H_3 heteroreceptors on cholinergic neurones, see Feuerstein (2008). Filled areas, ventricular system; dotted areas (*deep grey*), commissural tracts; dotted areas (*light grey*), glands

slices and synaptosomes, H_3 receptor activation leads to an inhibition of the synthesis of histamine (Arrang et al. 1987a). Moreover, there is evidence that H_3 receptors in the brain serve additional functions and are also located postsynaptically (Pollard et al. 1993; Lundius et al. 2010; Ellenbroek and Ghiabi 2014).

A salient property of native H_3 receptors is their constitutive activity. This has been shown by Rouleau et al. (2002) for rat brain cortex membranes with native (and recombinant) H_3 receptors, using the ^{35}S-GTPγS binding assay, i.e. a method in which G protein activation is measured (Strange 2010; first used for the H_3 receptor by Clark and Hill 1996). The constitutive activity of the H_3 receptors is unique since they occur in low density in the brain only and the maximum increase in ^{35}S-GTPγS binding is low as well (10–20%). By contrast, for native CB_1 receptors with their high binding density and their high amount of agonist-stimulated ^{35}S-GTPγS binding, the constitutive activity is not really surprising (Table 1). The constitutive activity of native H_3 receptors explains previous results obtained in rat brain cortex synaptosomes (Garbarg et al. 1992) in which thioperamide increased histamine release. At that time, thioperamide was believed to be a neutral H_3 receptor antagonist (Arrang et al. 1987b), and this property excludes a facilitatory effect in synaptosomes since in this type of preparation, unlike in slices, an accumulation of histamine in the biophase of the presynaptic H_3 receptors cannot build up. If thioperamide is however an inverse agonist, a facilitatory effect on histamine release will also be expected for a synaptosomal preparation.

2.3 Pharmacology

Characterization of H_3 receptors was facilitated by the increasing availability of powerful drug tools; only few compounds will be described here (Fig. 4). Agonists share the imidazole moiety with histamine. N^{α}-Methylhistamine is potent but unselective (Arrang et al. 1983), *R*-α-methylhistamine is potent and selective (Arrang et al. 1987b), and imetit is even more potent than the latter (Garbarg et al. 1992). Proxyfan is a protean drug, which, depending on the experimental model, behaves as a full or partial agonist, neutral antagonist or partial or full inverse agonist (Gbahou et al. 2003). Many inverse H_3 receptor agonists (which were originally believed to be neutral antagonists) have been synthesized (reviewed by Łażewska and Kieć-Kononowicz 2014; Sadek and Stark 2016; Panula et al. 2015). Thioperamide was the first representative with selectivity for H_3 over H_1 and H_2 receptors (Arrang et al. 1987b) but due to its toxic thiourea moiety was not interesting for further development (Tozer and Kalindjian 2000). Ciproxifan is selective and highly potent but still possesses an imidazole ring, which originally has been found to be a prerequisite for potent H_3 receptor antagonism/inverse agonism (Tozer and Kalindjian 2000). Imidazole-bearing drugs are, however, particularly prone to drug interactions involving the cytochrome P450 system (Slater et al. 1999). Another two shortcomings of ciproxifan became evident when the H_3 and H_4 receptors were cloned. Thus, ciproxifan is less potent at the

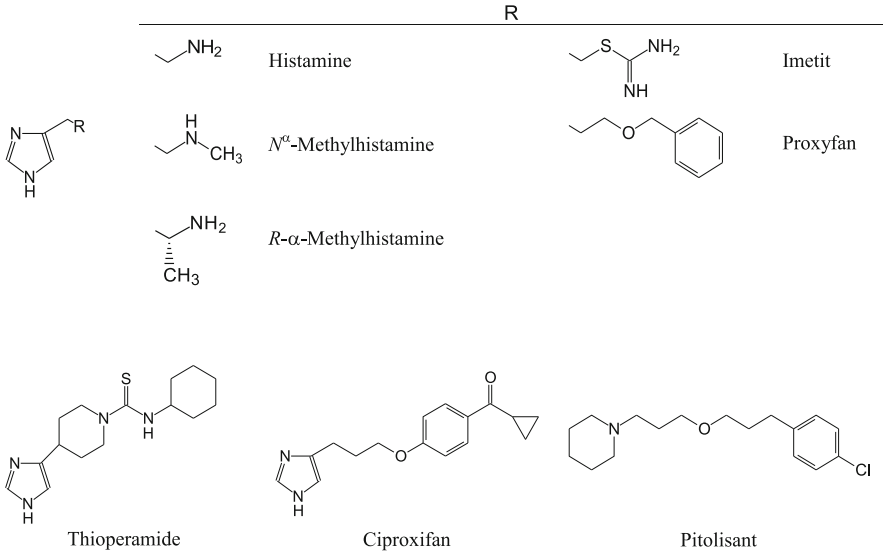

Fig. 4 Structures of selected histamine H_3 receptor ligands. The three compounds in the bottom part are inverse agonists. The other compounds are agonists except for proxyfan, which is a protean agonist (for further details, see text)

human when compared to the rodent H_3 receptor (Ligneau et al. 2000) and has a marked affinity for H_4 receptors (Panula et al. 2015). The three disadvantages could be overcome by pitolisant which contains a piperidine moiety (Meier et al. 2001; Liedtke et al. 2003; Schwartz 2011); its affinity for human H_3 receptors exceeds that for the other three histamine receptor types at least 200-fold (Panula et al. 2015).

3 Function of H_3 Receptors

For a better understanding of the role played by the H_3 receptors in the brain, it is mandatory to evaluate behavioural studies. H_3 receptors influence numerous central functions, and due to the limited space, we have restricted ourselves to four types of behaviour which hold some promise for therapeutic exploitation. Two scientific approaches were used for behavioural experiments, namely, drugs targeting H_3 receptors (which most frequently were studied in rodents) and H_3 receptor knockout mice. Since H_3 receptors serve as autoreceptors leading to an inhibition of histamine release, the influence of the histamine precursor His and of inhibitors of the histamine-forming and histamine-degrading enzymes His decarboxylase (HDC) and histamine *N*-methyltransferase (HNMT), respectively, was also considered (Fig. 5). HDC and HNMT knockout mice have also been created, but behavioural data with HNMT knockout mice have so far not been published (Schneider et al. 2014b). Targeting H_1 and H_2 receptors by using drugs or knockout mice is also of major interest; the H_2 receptor attracted less attention than the H_1 receptor.

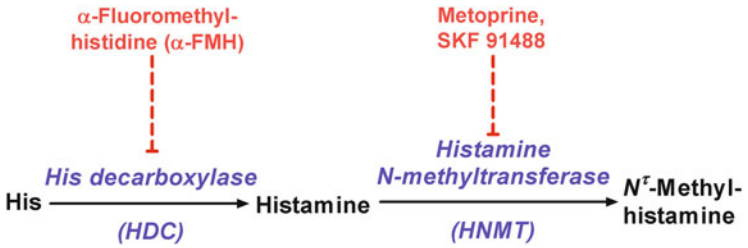

Fig. 5 Synthesis and catabolism of histamine in the brain, involved enzymes (*blue*) and their inhibitors (*red*). His, histidine

H_4 receptors will not be considered here since a systematic behavioural study on H_4 receptor knockout mice is still missing (Schneider et al. 2014b). The use of both drugs and knockout mice is of utmost importance since the approaches are to some extent complementary. Knockout mice although being highly selective may lead to compensatory changes since the knockout is permanent (inducible knockout mice targeting the histamine system would be of utmost importance). By contrast, drugs may have off-target effects, but the time of exposure can be tailored easily.

3.1 Sleep-Wake Regulation

The sleep-wake regulation in the cerebral cortex is influenced by two ascending systems, a ventral one (including the reticular activating system) and a dorsal one (implicating the hypothalamus). Both systems have direct and indirect parts, the latter ones involving the thalamus. A series of neurotransmitters play a role including amino acids (glutamate and GABA) and monoamines (including noradrenaline and serotonin). A particularly important role is played by histamine and the peptide orexin, which serve complementary functions. The former maintains the waking state, whereas the latter influences related motor activity and other behaviours accordingly (reviewed in Jones 2005; Lin et al. 2011). Histaminergic neurones show a tonical and specific pattern of firing during the wake state. If the formation of histamine is blocked by α-FMH or is impossible (HDC knockout mice), waking is decreased. If, on the other hand, histaminergic transmission is increased by the HNMT inhibitor SKF91488, waking is facilitated. The effect of histamine on waking is related to activation of H_1 receptors; this fact also explains why centrally active H_1 receptor antagonists (the classical antihistamines) have sedating properties. An involvement of H_2 receptors is less clear since controversial data have been obtained (see below and review by Lin et al. 2011).

H_3 receptors represent a twofold brake against the wake-promoting activity of the histaminergic system. Since they are constitutively active, they will exhibit an inhibitory effect even in the absence of histamine; their inhibitory influence will be further increased if histamine is released in their biophase. The role of the H_3 receptor system in sleep-wake control is now well established since a variety of

studies have been conducted in which the wakefulness has been quantified by electroencephalograms (EEG) and related methods; for the sake of simplicity, the term "wakefulness" will be used here. The facilitatory effect of inverse H_3 agonists like thioperamide, ciproxifan and pitolisant on wakefulness has been described for cats (Lin et al. 1990), guinea pigs (McLeod et al. 1998), rats (Lamberty et al. 2003) and mice (Parmentier et al. 2002; Toyota et al. 2002). H_3 agonists like *R*-α-methylhistamine, imetit and Sch 50971 had an opposite effect in cats (Lin et al. 1990), guinea pigs (McLeod et al. 1998), rats (Monti et al. 1991; Lamberty et al. 2003) and mice (Parmentier et al. 2007). In two studies (Lin et al. 1990; McLeod et al. 1998), the effect of thioperamide was counteracted (1) by the H_3 agonist *R*-α-methylhistamine and (2) by an H_1 antagonist.

Particularly sophisticated studies could be performed when knockout mice for the HDC and the histamine receptor subtypes became available. As expected, the facilitatory effect of H_3 inverse agonists and the inhibitory effect of H_3 agonists on wakefulness did no longer occur in H_3 knockout mice (Parmentier et al. 2002; 2007; Toyota et al. 2002; Gondard et al. 2013). The fact that inverse H_3 agonists no longer had a facilitatory effect in HDC knockout mice suggests that they act on H_3 receptors on histaminergic neurones (autoreceptors) (Parmentier et al. 2002, 2007). The facilitatory effect of the inverse H_3 agonist ciproxifan was absent in H_1 receptor knockout mice (Parmentier et al. 2007), confirming the results described above with H_1 and H_3 receptor ligands and once again underpinning the "functional antagonism" between both histamine receptor subtypes. On the other hand, ciproxifan retained its effect in H_2 knockout mice (Parmentier et al. 2007). The latter finding fits well to the study by Monti et al. (1990) in which the H_2 antagonist zolantadine (which penetrates the blood-brain barrier) had no effect on the sleep-wake cycle in rats. On the other hand, ranitidine, another H_2 antagonist, given intracerebroventricularly (i.c.v.), increased the slow-wave sleep of cats (i.e. favoured sleeping), suggesting an involvement of H_2 receptors (Lin 2000).

The beneficial effect of H_3 receptor inverse agonists on wakefulness (and on other functions of the CNS; see later) prompted several companies to develop such compounds as new drugs, e.g. for the treatment of narcolepsy, a rare neurological disease characterized by excessive daytime sleepiness and abnormal rapid eye movement (REM) sleep. The disorder is related to the destruction of most of the hypothalamic orexin (hypocretin)-producing neurones (Liblau et al. 2015), i.e. exactly of that peptide which like histamine plays a major role in the sleep-wake cycle (see above). Preclinical studies on Doberman dogs and orexin$^{-/-}$ mice, two experimental models of narcolepsy, revealed beneficial effects of several H_3 receptor inverse agonists (Bonaventure et al. 2007; Lin et al. 2008). The H_3 inverse agonist pitolisant inhibited the direct transition from waking to REM sleep in orexin$^{-/-}$ mice (the so-called narcoleptic episodes), whereas modafinil, the standard anti-narcoleptic drug, failed to do so (Lin et al. 2008). Pitolisant increases cortical histamine release in orexin$^{-/-}$ mice (Lin et al. 2008), and there is no doubt that this effect is related to the constitutive activity of the H_3 autoreceptors. In addition, pitolisant leads to an increased noradrenaline release which is believed to have a beneficial effect on the cataplexy (Nishino et al. 2000; cataplexy, occurring in 9 of

10 narcoleptic patients, is defined as the sudden loss of muscle tone triggered by strong emotions). Although the increase in noradrenaline release may be due to an indirect mechanism (e.g. may involve interneurones), it might also be related to constitutively active H_3 heteroreceptors on the noradrenergic nerve endings as suggested by own data on isolated cortical slices of wild-type mice (Fig. 2). H_3 inverse agonists were also compared to other anti-narcoleptic drugs. In mice, behavioural excitation and sleep rebound occurring with stimulants like amphetamine were not shared by H_3 inverse agonists and by modafinil (reviewed in Lin et al. 2011). Moreover, pitolisant unlike modafinil did not show drug abuse liability in in vivo rodent and primate models (Uguen et al. 2013).

A series of H_3 receptor inverse agonists has entered clinical studies including pitolisant (for review, see Lin et al. 2011; Schwartz 2011; Łażewska and Kieć-Kononowicz 2014; Panula et al. 2015; clinicaltrials.gov). Pitolisant was compared to placebo and modafinil in a double-blind, randomized, parallel group-controlled multicentre study in which the alteration of the Epworth Sleepiness Scale served as the primary endpoint (Dauvilliers et al. 2013). Pitolisant was superior to placebo in this respect and exhibited non-inferiority and was well tolerated when compared to modafinil. Moreover, the half-time of pitolisant of 11 h (Schwartz 2011) is favourable since the effect on wakefulness of one oral dose in the morning ceases during the night, thereby avoiding insomnia. In March 2016, the European Commission granted a marketing authorisation for pitolisant (Wakix^R) for the treatment of narcolepsy with or without cataplexy; Wakix^R received an orphan designation since narcolepsy belongs to the rare diseases (European Medicines Agency 2016). The recommendation is based on four clinical studies encompassing 466 patients. Pitolisant is the first-in-class drug that acts on H_3 receptors in the brain. This drug has also been studied in patients with excessive daytime sleepiness associated with Parkinson's disease (Lin et al. 2011; Schwartz 2011; clinicaltrials.gov) and other disorders (Leu-Semenescu et al. 2014).

3.2 Learning and Memory

If one searches for "learning, memory" and one of the four transmitters dopamine, GABA, glutamate or serotonin in the PubMed database, more than 2000 entries will appear in each case, whereas less than 350 entries will be found for the combination with histamine. Its relevance for cognitive functions was, e.g. shown in rats in which i.c.v. injection of α-FMH led to a memory deficit, which was ameliorated by histamine i.c.v. (Chen et al. 1999). In a mouse model with impaired learning and memory, metoprine given subcutaneously (s.c.) led to an improvement, which was also obtained by i.c.v. administration of H_1 receptor agonists. The beneficial effects of metoprine and the H_1 agonists were counteracted by the H_1 antagonist mepyramine given intraperitoneally (i.p.) (Malmberg-Aiello et al. 2000). The relevance of histamine for learning and memory is further highlighted by experiments on H_1, H_2 and HDC knockout mice. The dementia occurring in H_1 knockout mice is particularly severe and includes an impaired novelty-induced alternation behaviour (Zlomuzica et al. 2008), temporal order memory (Zlomuzica

et al. 2013), episodic-like memory (Dere et al. 2008) and spatial memory (Zlomuzica et al. 2009). H_1 and H_2 knockout mice resemble each other in that they exhibit an impaired spatial learning and object recognition but an improved acquisition of auditory and contextual freezing; moreover, long-term potentiation is attenuated in either mutant (Dai et al. 2007). Finally, HDC knockout mice show a combination of impaired (object discrimination) and improved cognitive paradigms (water-maze performance) (Dere et al. 2003).

The effect of H_3 inverse agonists on cognitive behaviour has been studied in rats and mice. The compounds were administered orally, i.p. or s.c. Rodents were not pretreated, or cognitive deficits were induced by dizocilpine, ketamine, pentetrazole or scopolamine, or senescence-accelerated mice were used. Ten inverse agonists with imidazole (thioperamide) or non-imidazole structure (including pitolisant) had a beneficial effect (for details, see Table 1 in Zlomuzica et al. 2016). In the studies of Miyazaki et al. (1995a; b), the procognitive effect of thioperamide was further increased by the H_2 antagonist zolantadine but counteracted by *R*-α-methylhistamine and mepyramine.

The effect of H_3 inverse agonists on cognitive function of humans was examined in a series of clinical studies. It is of interest in this context that the density of H_1 receptor binding sites in some brain areas of patients suffering from Alzheimer's disease was found to be correlated with the severity of cognitive symptoms (reviewed by Zlomuzica et al. 2016). In patients with mild-to-moderate Alzheimer's disease, ABT-288 had no effect (Haig et al. 2014), whereas another H_3 inverse agonist, GSK239512, moderately improved some, but not all cognitive parameters (Nathan et al. 2013; Grove et al. 2014). However, replication of the results with a higher number of patients is mandatory. H_3 inverse agonists were also examined in schizophrenic patients with cognitive impairment. MK-0249 was ineffective in this respect (Egan et al. 2012); a study with pitolisant has not yet been completed (clinicaltrials.gov). Finally, patients with attention-deficit hyperactivity disorder (ADHD) were treated with H_3 inverse agonists. The final outcome of an early study with GT-2331 has never been disclosed (Tozer and Kalindjian 2000); the recent study with bavisant (JNJ-31001074) did not yield a positive result (Weisler et al. 2012).

3.3 Susceptibility to Seizures

Histamine is like an endogenous anticonvulsive principle (Iinuma et al. 1993; Haas et al. 2008; Bhowmik et al. 2012). The evidence is based on experiments in which seizure susceptibility of animals was attenuated by measures that increase brain histamine including administration of His (Chen et al. 2002; Yawata et al. 2004) or metoprine (Tuomisto and Tacke 1986; Yawata et al. 2004). By contrast, a higher susceptibility to seizures occurred when histamine synthesis was inhibited (by α-FMH; Jin et al. 2007) or impossible (HDC knockout mice; Chen et al. 2003), in mast cell-deficient mice (Chen et al. 2003) or when one of the five groups of histaminergic cell groups (E2) in the posterior hypothalamus was lesioned (Jin

et al. 2007). The protective effect of histamine involves H_1 receptors as suggested by the clinical experience that H_1 receptor antagonists can induce seizures, particularly in children (Iinuma et al. 1993; Yokoyama 2001; Simons 2004). A proconvulsant effect of H_1 antagonists was also observed in animals (listed by Haas et al. 2008). Moreover, H_1 receptor knockout mice show an increased seizure susceptibility (Chen et al. 2003; Hirai et al. 2004; Kukko-Lukjanov et al. 2010, 2012). Interesting enough, also the H_2 receptor antagonist famotidine can induce seizures in humans (von Einsiedel et al. 2002). The role of H_2 receptors in seizures is so far unclear since experiments with selective ligands on animals are rare (Chen et al. 2002, see later) and experiments on H_2 knockout mice are completely missing. By the way, it is surprising at first glance that endogenous histamine has a *stimulatory* effect on the waking state as opposed to an *inhibitory* effect on seizure susceptibility. The reason for this discrepancy has not been fully elucidated, but the protective effect of histamine against seizures may be related to its facilitatory effect on GABAergic interneurones and its inhibitory effect on the glutamatergic principal neurones of the hippocampus. The latter effects most probably outweigh its stimulatory influence on cortical activity (for a more detailed discussion, see Haas et al. 2008).

Since histamine, via H_1 receptors, serves as an endogenous anticonvulsive agent, one should expect that H_3 inverse agonists have a similar effect. A series of H_3 inverse agonists has been administered i.p. to rodents, and maximal electroshock (MES)-, pentetrazole (PTZ)- and strychnine (STR)-induced seizures were considered, or amygdaloid-kindled rats or EL mice were examined. A beneficial effect was obtained for 6 compounds including the imidazoles thioperamide and clobenpropit (for details of the studies, see Bhowmik et al. 2012) and the non-imidazoles pitolisant and DL77 (Sadek et al. 2016). The effect of thioperamide and clobenpropit was counteracted by the H_3 receptor agonists *R*-α-methylhistamine or immepip (reviewed by Bhowmik et al. 2012), and the effect of DL77 was antagonized by *R*-α-methylhistamine and mepyramine (Sadek et al. 2016). However, H_3 inverse agonists did not lead to positive results in all epilepsy models (Bhowmik et al. 2012; Sadek et al. 2016). In the investigation by Sturman et al. (1994), thioperamide even increased the severity of clonic convulsions of picrotoxin-induced seizures in mice. Despite those inconsistencies, H_3 inverse agonists may be of interest as potential anticonvulsive drugs in humans. One has to consider that they have neuroprotective (Bhowmik et al. 2012) and cognition-enhancing properties (see above) which may be beneficial since the disease process and/or other anticonvulsant drugs may have detrimental effects on brain function.

3.4 Food Intake

Food intake is regulated by transmitters, neuropeptides and hormones in brain areas like the nucleus tractus solitarii (NTS) and the periventricular (PVN) and ventromedial nucleus (VMN); the latter two regions are located in the hypothalamus like

the tuberomammillary nucleus which contains the histaminergic cell bodies. Food intake is reduced, e.g. by nesfatin-1, corticotropin releasing hormone (CRH), thyreotropin releasing hormone (TRH), glucagon-like-peptide 1 (GLP-1), leptin and oestradiol but increased, e.g. by endocannabinoids, neuropeptide Y (NPY) and proopiomelanocortin (reviewed in Schneider et al. 2014a). The anorectic role of histamine has been shown in a variety of experiments on animals, e.g. food intake has been reduced by injection of histamine directly into relevant brain areas or the ventricular system or by systemic administration of compounds leading to an increase in endogenous histamine (His or metoprine). Histamine inhibits appetite rather than being a satiety signal. H_1 receptors are involved. Thus, H_1 agonists decreased, whereas H_1 antagonists increased food intake (reviewed in Passani et al. 2011; Tabarean 2016). The role of H_1 receptors is also underpinned by findings on H_1 knockout mice. Thus, the effect of anorectic compounds (nesfatin-1 i.c.v., TRH i.c.v., leptin i.p., oestrogen i.p.) on food intake was decreased or abolished, whereas the effect of the orexigenic NPY i.c.v. was increased (Table 1 in Schneider et al. 2014a). Moreover, H_1 knockout mice have an increased body weight although this becomes evident only at the age of 28–30 weeks (Masaki et al. 2004).

To elucidate the role of H_3 receptors played in food intake, experiments with H_3 ligands and/or H_3 knockout mice have been carried out. As expected, the H_3 agonists imetit and *R*-α-methylhistamine given i.p. increased food intake in rats and mice, respectively (Clapp and Luckman 2012; Jørgensen et al. 2005). By contrast, when given orally or i.p. to rodents, 11 H_3 antagonists (including ciproxifan and thioperamide) reduced food intake, and 9 (including ciproxifan) diminished body weight; animals received a standard or high-fat diet (studies listed in Table 1 of Provensi et al. 2016). A reduction of food intake was also observed in pigs and obese rhesus monkeys that received an intragastric and s.c. administration of the H_3 antagonist NNC 38-1202, respectively (Malmlöf et al. 2007). Moreover, in rats thioperamide i.p. potentiated the effect of metoprine i.p. (Lecklin and Tuomisto 1998), whereas the H_1 antagonist chlorpheniramine i.p. abolished the effect of thioperamide i.c.v. (Ookuma et al. 1993). In the same species, the protean H_3 ligand proxyfan i.p. blocked both the effect of the H_3 agonist immepip i.p. and of thioperamide i.p., which increased and decreased food intake, respectively (Clapp and Luckman 2012). In the latter study, thioperamide and proxyfan did not affect food intake by themselves.

However, some studies do not fit into this picture. H_3 agonists or inverse agonists administered systemically or i.c.v. to rodents had no effect on food intake in few studies (listed in Table 1 of Provensi et al. 2016). Moreover, the increase in food intake elicited by i.c.v. administration of histamine to sheep was not counteracted by *R*-α-methylhistamine (Rahmani and Ingram 2007). Yoshimoto et al. (2006) even found that imetit *decreased* and thioperamide *increased* food intake; both effects were abolished in H_3 knockout mice. Finally, H_3 knockout mice show an obese phenotype. The reasons for the discrepancies may in part be explained by the fact that different orexigenic pathways were involved. The unexpected increase in body

weight occurring in H_3 knockout mice may point to adaptive changes occurring when the H_3 receptor protein is lacking ab initio.

The favourable effect of inverse H_3 agonists in many rodent studies prompted scientists to examine the effect of betahistine on body weight in obese humans. This drug (which is indicated for the treatment of Menière's disease; Wright 2016) combines H_1 agonism (Seifert et al. 2013) and inverse H_3 agonism (Gbahou et al. 2010), both of which are known to decrease food intake. The results (listed in Table 2 of Provensi et al. 2016) were disappointing. On the other hand, when in a double-blind study betahistine was administered to patients treated with the atypical antipsychotic olanzapine, the weight gain was lower than in the placebo group (Poyurovsky et al. 2013). Although the data appear interesting, they are based on a very low number of patients only. Moreover, since betahistine was given in combination with the selective noradrenaline reuptake inhibitor reboxetine, the role played by the latter drug has to be further investigated.

4 Conclusions

Histamine H_3 receptors inhibit (1) histamine release from the histaminergic nerve endings (presynaptic autoreceptor), (2) impulse flow along the histaminergic neurones (somadendritic autoreceptor) and (3) histamine synthesis. Although H_3 receptors occur only at a small density in the brain, they have the unique property that they are constitutively active. These receptors already work in the absence of endogenous histamine but are the more active if they are activated by endogenous histamine. In other words, they represent a double brake against activation of postsynaptic H_1 and H_2 receptors by endogenous histamine. Determination of the role played by the H_3 autoreceptors in the brain was facilitated by the availability of selective H_3 receptor ligands and H_3 receptor knockout mice. Inverse H_3 agonists including thioperamide, ciproxifan and pitolisant increase wakefulness, ameliorate learning and memory, inhibit some types of epileptic seizures and decrease food intake and weight gain in animals, particularly in rodents. These effects are related to the activation of postsynaptic H_1 (and in the case of learning and memory also of postsynaptic H_2) receptors by endogenously released histamine and are opposite in direction to typical side effects elicited by the brain-penetrating H_1 antagonists (see, e.g. Table 4 in the review by Simons 2004). H_3 receptor inverse agonists have also been examined in clinical studies and are suited for the treatment of excessive daytime sleepiness. Pitolisant (Wakix^R) has received a marketing authorisation from the European Commission for the treatment of narcolepsy with or without cataplexy in 2016. Besides H_3 autoreceptors, also presynaptic H_3 heteroreceptors were described, i.e. receptors leading to the inhibition of serotonin, noradrenaline, dopamine, glutamate, GABA and perhaps acetylcholine release from their respective neurones. The role played by the H_3 heteroreceptors is poorly understood, and experiments with knockout mice in which the H_3 receptor deficiency is restricted to single neuronal systems are needed.

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Clinical Development of Histamine H_4 Receptor Antagonists

Robin L. Thurmond, Jennifer Venable, Brad Savall, David La, Sandra Snook, Paul J. Dunford, and James P. Edwards

Contents

Abstract

The discovery of the histamine H_4 receptor (H_4R) provided a new avenue for the exploration of the physiological role of histamine, as well as providing a new drug target for the development of novel antihistamines. The first step in this process was the identification of selective antagonists to help unravel the pharmacology of the H_4R relative to other histamine receptors. The discovery of the selective H_4R antagonist JNJ 7777120 was vital for showing a role for the H_4R in inflammation and pruritus. While this compound has been very successful as a tool for understanding the function of the receptor, it has drawbacks, including a short in vivo half-life and hypoadrenocorticism toxicity in rats and dogs, that prevented advancing it into clinical studies. Further research let to the discovery of JNJ 39758979, which, similar to JNJ 7777120, was a potent and selective H_4R antagonist and showed anti-inflammatory and anti-pruritic activity preclinically. JNJ 39758979 advanced into human clinical studies and showed efficacy in reducing experimental pruritus and in patients with atopic dermatitis. However, development of this compound was terminated due to the occurrence of drug-induced agranulocytosis. This was overcome by developing another H_4R

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antagonist with a different chemical structure, toreforant, that does not appear to have this side effect. Toreforant has been tested in clinical studies in patients with rheumatoid arthritis, asthma, or psoriasis. In conclusions there have been many H_4R antagonists reported in the literature, but only a few have been studied in humans underscoring the difficulty in finding ligands with all of the properties necessary for testing in the clinic. Nevertheless, the clinical data to date suggests that H_4R antagonists can be beneficial in treating atopic dermatitis and pruritus.

Keywords
Antihistamines • Atopic dermatitis • Inflammation • JNJ 39758979 • JNJ 7777120 • Pruritus • Rheumatoid arthritis • Toreforant

1 Introduction

The first reports of the identification of the histamine H_4 receptor (H_4R) were published in 2000–2001. It was the fourth member of the histamine receptor family and, along with the histamine H_1, H_2, and H_3 receptors, mediates the physiological functions of histamine (Panula et al. 2015). Discovery of this receptor provided a new avenue to explore histamine's biologic role and spurred basic research into the function of the receptor. This work has resulted in the testing of H_4R antagonists in the clinic (Table 1) and some data have recently emerged. In this review we will give a historical account of development of H_4R ligands at Janssen Research & Development, LLC.

The H_4R was discovered by identification of a genomic sequence that had the signature of a G-protein coupled receptor and was shown to bind histamine (Thurmond 2015). Profiling the activity of known histamine receptor ligands indicated that this receptor exhibited unique pharmacology and thus was named the histamine H_4 receptor. The initial pharmacological characterization of the receptor indicated that many previously characterized histamine H_3 receptor ligands, such as thioperamide, were also ligands for the H_4R. Thioperamide was initially described as a potent and selective histamine H_3 receptor antagonist, but the initial pharmacological characterization of the H_4R showed that it was also a potent H_4R antagonist (Arrang et al. 1987; Liu et al. 2001). This was not surprising given the high homology between the two receptors. Subsequently, 4-methylhistamine, which was known as a selective histamine H_2 receptor agonist, was shown to also be a potent H_4R agonist (Durant et al. 1975; Lim et al. 2005). While the availability of these ligands proved useful in some of the early characterization of the receptor (Buckland et al. 2003; Hofstra et al. 2003; Takeshita et al. 2003; Bell et al. 2004), it was clear that ligands selective for the H_4R would be needed to uncover its specific pharmacology.

Table 1 Clinical studies with H_4R antagonists

Indication	Compound	Results
Histamine-induced itch	JNJ 39758979	JNJ 39758979 reduced histamine-induced itch
Bronchial allergen challenge	ZPL-389	Not reported
Allergic rhinitis	UR-63325	Not reported
Atopic dermatitis	JNJ 39758979	Trend for efficacy in EASI. Nominally statistically significant reduction in pruritus
	ZPL-389	Nominally statistically significant reduction in EASI
Rheumatoid arthritis	Toreforant	Toreforant 100 mg/day showed reduction in DAS28 and in ACR response rates. Follow-up study at 3, 10 and 30 mg/day showed no efficacy
Asthma	Toreforant	Not reported
	JNJ 39758979	Not reported
Psoriasis	ZPL-389	Not reported
	Toreforant	Not reported

2 Early Selective H_4 Receptor Antagonists

In order to identify novel starting points for medicinal chemistry efforts to develop potent and selective H_4R ligands, a high throughput screen of a large compound library was conducted. The screen looked for compounds that could inhibit histamine binding to membranes expressing the human H_4R. This assay yielded several lead compounds including an indolylpiperazine (Fig. 1; Compound 1) that was a potent ligand for the H_4R with a K_i of 38 nM (Jablonowski et al. 2003). The subsequent medicinal chemistry effort focused on evaluation of various substituents on the indole core while maintaining the optimal *N*-methylpiperazine as the terminus. Small substituents in the 5 and 7-positions were well tolerated leading to many compounds with high affinity for the H_4R (Jablonowski et al. 2003). This work cumulated in the identification of JNJ 7777120 (Fig. 1) that had a K_i of 4.5 nM versus the human receptor and demonstrated functional antagonism with a pA_2 of 8.1 with at least 1,000-fold selectivity over the histamine H_1, H_2, or H_3 receptors and no cross-reactivity against 50 other targets (Thurmond et al. 2004). It was also a high affinity antagonist for the mouse and rat H_4R (Table 2). The proper characterization of the pharmacology of ligands in species besides human is absolutely crucial for interpreting preclinical data. This is especially important when dealing with data from preclinical animal models of human diseases where one must know the affinity, pharmacological action (agonist vs. antagonist), and compound levels in the species where the model is run. This information is essential in determining whether the effects seen in the animal model would also be seen in humans. In that same vein, the pharmacology also needs to be understood in the species being used

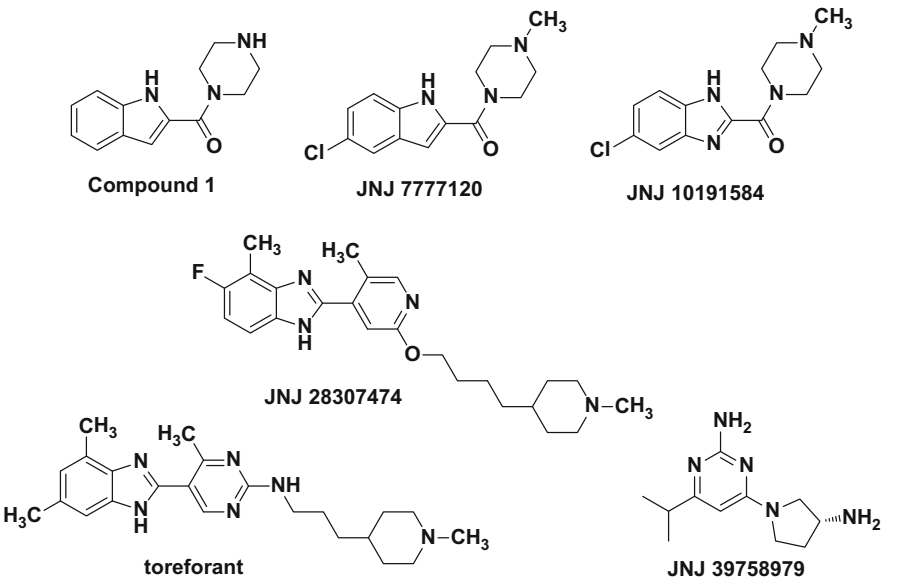

Fig. 1 Compound structures

Table 2 K_i (nM) at the various histamine receptors[a]

	JNJ 7777120	JNJ 10191584	JNJ 28307474	JNJ 39758979	Toreforant
Human H_4R	4.1	27	4.9	12.5	8.4
Mouse H_4R	4.6	55	109	5.3	307
Rat H_4R	2.6	97	87	188	9.3
Dog H_4R	79	630	62	>10,000	680
Monkey H_4R	32	199	ND	25	10.6
Human H_1R	>10,000	>10,000	2,501	>1,000	>10,000
Human H_2R	>1,000	>1,000	>1,000	>1,000	>1,000
Human H_3R	5,125	7,000	159	1,043	215

[a]*ND* not determined

for the toxicology studies required before testing in humans in order to make sure any potential safety issues are uncovered. For example, it would be inappropriate to use preclinical safety data to justify a human clinical study for a compound that is an antagonist in the toxicology species, but an agonist in humans.

As the first potent and selective H_4R antagonist, JNJ 7777120 has become one of the key standard ligands to define H_4R activity both in vitro and in vivo. In particular this ligand provided the first evidence that the H_4R was involved in inflammation in vivo. Based on the expression profile of the receptor, the activity of JNJ 7777120 was tested in a number of preclinical inflammation models to

elucidate its potential anti-inflammatory activity. One of the first models to show effects was a mouse peritonitis model. Zymosan, a toll-like receptor agonist, induces neutrophil influx within 4 h of being injected into the peritoneum. Pretreatment with JNJ 7777120 led to a reduction in the neutrophil influx, indicating an anti-inflammatory effect (Thurmond et al. 2004). Efficacy was also seen when chemokine (C-X-C motif) ligand-1 (CXCL1) or sodium urate crystals were used to induce peritonitis, but not when thioglycollate was used (Thurmond et al. 2004 and unpublished data). These results confirmed other results in peritonitis models with non-selective H_4R ligands (Takeshita et al. 2003). Anti-inflammatory efficacy was also seen in a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute colitis model in rats. Here treatment with JNJ 7777120 led to a reduction in the colonic lesion area as well as reduced tissue myeloperoxidase and TNF-α levels (Varga et al. 2005). Since these initial observations, efficacy with JNJ 7777120 has subsequently been demonstrated in models of asthma, pulmonary fibrosis, pleurisy, dermatitis, anaphylaxis, pruritus, lipopolysaccharide (LPS)-induced inflammation, allergic rhinitis, allergic conjunctivitis, experimental allergic encephalomyelitis, and pain (Dunford et al. 2006, 2007; Smith et al. 2007; Nakano et al. 2009; Rossbach et al. 2009, 2011; Takahashi et al. 2009; Cowden et al. 2010a, b, 2013; Seike et al. 2010; Suwa et al. 2011; Beermann et al. 2012; Matsushita et al. 2012; Ohsawa and Hirasawa 2012; Ballerini et al. 2013; Somma et al. 2013; Ahmad et al. 2014, 2015; Mahapatra et al. 2014; Pini et al. 2014; Rosa et al. 2014; Lucarini et al. 2016; Wang et al. 2016).

Keeping in mind an important principle of pharmacology, it was important to not misinterpret the function of the H_4R based on the activity on a single ligand since the full specificity of that ligand may be unknown (see, for example, thioperamide specificity prior to the discovery of the H_4R) or, alternatively, its activity as an antagonist versus agonist may vary depending on conditions. One way to support the findings with a purported antagonist like JNJ 7777120 was to show that H_4R-deficient mice have a similar phenotype in the model of question. Indeed this has been shown for mouse asthma, dermatitis, LPS-induced inflammation, colitis, experimental allergic encephalomyelitis, and pruritus models, which supports the conclusions that the effects seen in vivo with JNJ 7777120 are due to antagonism of the H_4R (Dunford et al. 2006, 2007; Cowden et al. 2010b, 2013; del Rio et al. 2012; Schirmer et al. 2015). Another way to support selectivity is to show similar effects with different H_4R antagonists. To this end the results seen with JNJ 7777120 have been replicated by other compounds in models of colitis, asthma, dermatitis, pain, and pruritus (Varga et al. 2005; Dunford et al. 2006, 2007; Coruzzi et al. 2007; Altenbach et al. 2008; Cowart et al. 2008; Liu et al. 2008; Cowden et al. 2010b, 2014; Hsieh et al. 2010; Shin et al. 2012; Savall et al. 2014; Thurmond et al. 2014).

While JNJ 7777120 is an excellent pharmacological tool for helping dissect the role of the H_4R in several disease states, it has limitations that preclude its development as a drug. One of these is that it is rapidly metabolized in vivo and therefore the pharmacokinetics are not appropriate for an oral human therapeutic or even for dosing in long-term animal models; even short term models may require very high doses or multiple dosing regimens. JNJ 7777120 has an oral

bioavailability of ~30% in rats and 100% in dogs with a half-life of ~3 h in both species (Thurmond et al. 2004). In mice the half-life was around 1–2 h. The compound appears to work best in acute challenge models such as the mouse asthma, pruritus, and atopic dermatitis models. However, in more chronic models where continuous inhibition of the receptor is likely required, JNJ 7777120 is less effective. One example of such a model is the mouse collagen-induced arthritis model where JNJ 7777120 has not been shown to be efficacious, but other H_4R antagonists have (Cowden et al. 2014).

One of the main metabolites of JNJ 7777120 is demethylation of the piperazine. However, this is also important for potency of the compound since removing the methyl group reduces the affinity for the human H_4R to 25 nM (Engelhardt et al. 2012). One strategy for improving metabolic stability was to modify the core indole of the molecule including replacing the pyrrole ring of the indole carboxamides for imidazole (Venable et al. 2005). This led to a series of potent and selective compounds that had similar properties with their indole counterparts, however, their metabolic profiles differed. One of these compounds was JNJ 10191584 (Fig. 1), which shared all of the structural features of JNJ 7777120 except that the C(3) CH group was replaced with a nitrogen atom. Like JNJ 7777120, this compound was a potent and selective H_4R antagonist with a K_i of 26 nM (Table 2) (Venable et al. 2005). It also behaves in a similar fashion to JNJ 7777120 in models of asthma and colitis (Table 3) (Varga et al. 2005; Dunford et al. 2006). This compound exhibited no improvement in pharmacokinetics in rats or mice (half-life ~1 h), but the human in vitro data suggested that it would have better human exposure compared to JNJ 7777120.

With the improved human pharmacokinetic predictions, preclinical toxicity studies were initiated with JNJ 10191584. Dose range finding studies were carried out in rats and dogs. In rats (5 animals of each sex per group) doses of 0, 100, 250, 500, 1,000 mg/kg/day, divided b.i.d, were given for 5 days. The main findings were dose dependent decreases in serum sodium and chloride, increases in serum potassium, and decreased sodium:potassium ratios (Table 4). In dogs (1 animal of

Table 3 Activity in preclinical disease models[a]

	KO	JNJ 7777120	JNJ 10191584	JNJ 28307474	JNJ 39758979	Toreforant
Asthma	Y	Y	Y	Y	Y	Y
LPS-induced inflammation	Y	Y	NT	Y	Y	Y
Dermatitis	Y	Y	NT	Y	Y	Y
Collagen-induced arthritis	Y	N	NT	Y	Y	Y
Neuropathic pain	NT	Y	NT	NT	Y	N
Colitis	NT	Y	Y	NT	NT	NT
Histamine-induced pruritus	Y	Y	NT	Y	Y	N

[a]*Y* activity seen, *N* tested but no activity, *NT* not tested, *KO* H_4R-deficient mice

Table 4 Rat serum electrolytes in a 5 day oral toxicity study with JNJ 10191584

Dose mg/kg/day divided b.i.d.	Male				Female			
	Na	Cl	K	Na:K[a]	Na	Cl	K	Na:K
0	147.4	99.60	6.434	22.8	144.6	100.12	7.070	20.4
100	146.0	99.18	7.374	19.8	143.2	98.48	7.096	20.2
250	145.3	99.20	6.952	20.9	143.6	98.04	7.330	19.6
500	144.2	97.92	7.066	20.4	141.1	96.48	7.35	19.2
1,000	141.6	96.52	8.488	16.7	139.9	92.88	7.840	17.8

Five animals of each sex per group
[a]*Na:K* serum sodium to potassium ratio

each sex per group) doses of 0, 20, 100, 200, 300 mg/kg/day, divided b.i.d., were given for 5 days. As with rats decreased serum sodium and chloride levels and elevated serum potassium values were evident in the high dose animals with a lower sodium:potassium ratio relative to control group animals (Table 5). In the dog, histopathology of the adrenal gland revealed diffuse necrosis of the zona glomerulosa (Fig. 2). No histopathologic lesions were apparent in the adrenal glands of rats. This characteristic pattern of serum electrolyte effects and lowered sodium: potassium ratios is consistent with hypoadrenocorticism (Klein and Peterson 2010). In the dog this was corroborated by the striking necrosis of the adrenal gland zona glomerulosa, the anatomic region of the adrenal gland where aldosterone is synthesized. The mineralocorticoid, aldosterone, is critical for maintaining serum electrolyte balance and decreased sodium:potassium ratios are a hallmark of aldosterone deficiency. The adrenal gland is an unusual target organ for xenobiotic toxicity. In this case, because the serum electrolyte changes were seen in two species, developed rapidly over 5 days of treatment, and could result in life threatening serum electrolyte perturbations in human subjects, the development of JNJ 10191584 was terminated. Subsequent testing of JNJ 7777120 indicated that it too caused comparable serum electrolyte effects in rats. However, as noted above, the structures of JNJ 10191584 and JNJ 7777120 are very similar and therefore it was unclear whether these effects were due to H_4R antagonism or to the compound structures. To address this, an H_4R antagonist was needed from a different structural class.

A review of the initial compound screening results revealed another potential chemotype that led to the discovery of JNJ 28307474 (Fig. 1). This class of pyridinyl benzimidazoles is structurally distinct from the indole chemotypes, but does maintain a basic amine (piperidine) that is important for the interaction with the H_4R. From a pharmacological perspective it was similar to JNJ 7777120 with a high affinity for the human H_4R (Table 2), however, it was less potent at the mouse H_4R and did have some affinity for the human H_3 receptor (Cowden et al. 2010b). As for JNJ 7777120, this compound showed activity in mouse models of asthma, atopic dermatitis, LPS-induced inflammation, and itch (Table 3) (Cowden et al. 2010b, 2013; Dunford, unpublished data). In contrast to JNJ 7777120, JNJ 28307474 demonstrated activity in the mouse collagen-induced arthritis model,

Table 5 Dog serum electrolytes in a 5 day oral toxicity study with JNJ 10191584

Dose mg/kg/day divided b.i.d.	Male				Female			
	Na	Cl	K	Na:K[a]	Na	Cl	K	Na:K
0	142.9	109.3	4.52	31.6	144.1	109.7	4.30	33.5
20	143.2	110.3	4.29	33.4	145.0	110.8	4.43	32.7
100	143.4	111.6	4.28	33.5	143.5	109.5	4.59	31.3
200	139.0	109.0	4.44	31.3	144.6	110.5	4.73	30.6
300	140.7	108.7	4.64	30.3	136.9	104.6	4.83	28.3

One animal of each sex per group
[a]*Na:K* serum sodium to potassium ratio

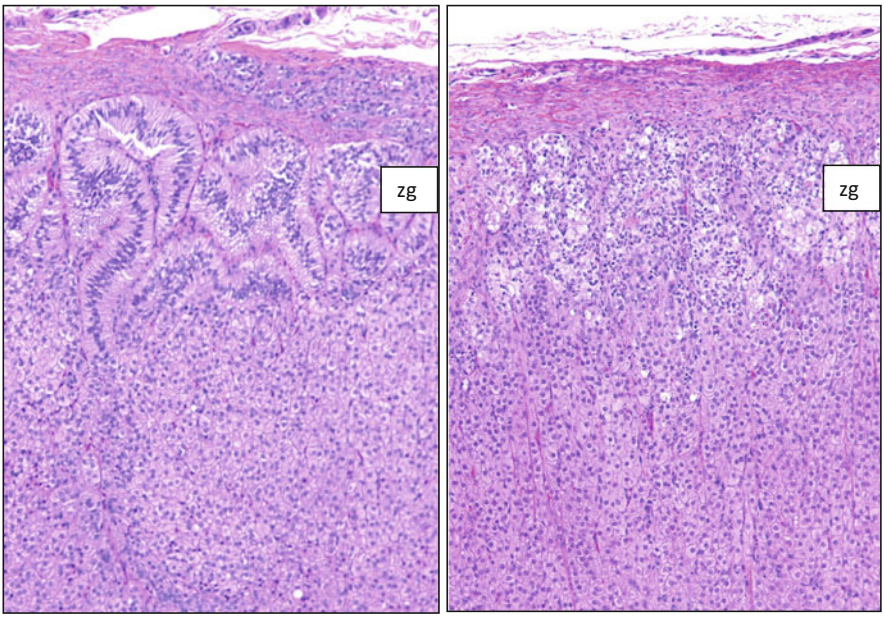

Fig. 2 Photomicrograph showing adrenal changes in a dog treated with JNJ 10191584. Adrenal glands of dog treated with vehicle (*left*) or 300 mg/kg/day JNJ10191584 (*right*). The normal canine zona glomerulosa (zg) is comprised of tall, organized epithelial cells with prominent eosinophilic cytoplasm and basally oriented nuclei. In the treated adrenal gland on the right the zona glomerulosa is diffusely necrotic

most likely due to the better half-life in the mouse compared to JNJ 7777120 that lacked this activity (half-life ~4–5 h). Importantly, no adrenal gland or associated toxicities were observed with JNJ 28307474 in rat and dog, supporting the conclusion that the findings seen with JNJ 10191584 and JNJ 7777120 were chemotype driven and not class effects related to the H_4R. However, a major drawback with

JNJ 28307474 was its inhibition of human ether-à-go-go-related gene (hERG) channel activity in vitro with an IC_{50} ~ 200 nM. This translated into in vivo QT-interval prolongation in both dogs (~10–15% increase) and monkeys (~30% increase). Prolongation of the QT-interval in humans can lead to serious arrhythmias and Torsade de Pointes. Therefore, this safety issue, and more specifically the lack of an estimated therapeutic window, led to the termination of the development of the compound.

3 Clinical Activity of JNJ 39758979

Once again a potential safety issue prompted the search for a different pharmacophore. Screening hits identified a tricyclic pyrimidine series as potential H_4R antagonists and led to the development of a series of both tricyclic and monocyclic aminopyrimidine antagonists (Savall et al. 2011). Out of this series emerged JNJ 39758979 (Fig. 1), one of the first H_4R antagonists to enter the clinic (Savall et al. 2014; Thurmond et al. 2014). JNJ 39758979 is a potent and selective H_4R antagonist with a K_i of 12.5 nM at the human H_4R (Table 2) and at least 80-fold lower affinity for the human histamine H_1, H_2, and H_3 receptors (Thurmond et al. 2014). Importantly, in contrast to JNJ 28307474, there was no inhibition of the human ether-à-go-go-related gene (hERG)-mediated K^+ current in transfected cells at concentrations up to 10 μM and no indication of cardiovascular effects in vivo (Savall et al. 2014). JNJ 39758979 exhibited excellent exposure, bioavailability, and half-life in mouse, rats, and dogs and this translated into excellent human pharmacokinetic properties (Savall et al. 2014; Thurmond et al. 2014). Consistent with other H_4R antagonists, JNJ 39758979 exhibited efficacy in preclinical models of asthma, dermatitis, pruritus, LPS-induced inflammation, and arthritis (Table 3) (Savall et al. 2014; Thurmond et al. 2014).

Preclinical safety testing indicated no issues that would preclude testing in humans (Thurmond et al. 2014). As with JNJ 28307474, no adrenal gland or associated toxicities were observed in any toxicology species tested, confirming that the findings with the previous compounds were related to their chemotype. Therefore, a phase 1 safety study was conducted with JNJ 39758979 in healthy human volunteers starting in September 2008. In the phase 1 study the only tolerability or safety issue noted was dose-dependent nausea thought to be due to local irritation, since it was reduced with an enteric coated formulation. The compound also exhibited excellent oral exposure with a long half-life. A pharmacodynamics assay was used to show that the compound inhibited the H_4R in vivo. This assay exploited the fact that when histamine is added to eosinophils a shape change is induced that can be detected by fluorescence activated cell sorting (FACS). Therefore, in the clinic blood was drawn from subjects after dosing with JNJ 39758979 and histamine was added. The inhibition of the eosinophil shape change was evidence that the compound was bound to the receptor and blocked its activation. Therefore, the dose-dependent inhibition of histamine-induced

eosinophil shape change observed suggested that JNJ 39758979 antagonized the H_4R in vivo.

H_4R antagonists have shown efficacy in several preclinical models of human disease and thus there were several possible avenues to explore in the clinic. One area of particular interest was pruritus since it has long been linked to histamine. In mice several different H_4R antagonists have been shown to inhibit scratching induced by histamine (Bell et al. 2004; Dunford et al. 2007; Cowart et al. 2008; Liu et al. 2008; Yamaura et al. 2009; Koenig et al. 2010; Shin et al. 2012; Savall et al. 2014). JNJ 39758979 given orally was efficacious in reducing histamine-mediated pruritus starting at doses of 5 mg/kg and higher, thus providing the rationale for testing the compound in humans (Savall et al. 2014). Histamine-induced pruritus in humans has been used for decades to study and compare the effect of antihistamines that target the histamine H_1 receptor. Injection of histamine into the skin of humans causes a perception of pruritus (i.e., the desire to scratch) within minutes of application. Mice also respond to the pruritic effects of histamine and therefore novel mechanisms can be tested in this model in mice and then directly translated into humans.

A clinical study was conducted to test the effect of JNJ 39758989 on pruritus in humans. Subjects were given a single dose of either JNJ 39758979, cetirizine (an histamine H_1 receptor antagonist), or placebo (Kollmeier et al. 2014). The use of JNJ 39758979 and cetirizine could definitively determine the relative roles for the histamine H_1 receptor and H_4R since the former only has affinity for the H_4R with no affinity for the histamine H_1 receptor and the later only exhibits histamine H_1 receptor antagonist activity with no affinity for the H_4R (Lim et al. 2005; Savall et al. 2014). Subjects were challenged with an intradermal injection of histamine one day before compound administration, to assess the baseline response, and then again 2 and 6 h after taking a dose of compound. At each of these times the subjects were asked to rate the itching sensation over a 10-min period. As predicted from the mouse model, JNJ 39758979 was able to significantly inhibit the pruritus induced by histamine to a similar extent as the positive control, cetirizine (Kollmeier et al. 2014). Of interest, JNJ 39758979 did not block the histamine-induced wheal response, although it was inhibited by cetirizine. This result was also predicted by the preclinical animal models where JNJ 7777120 was not able to block histamine-induced edema formation, whereas a histamine H_1 receptor antagonist was effective (Thurmond et al. 2004). Therefore, the specificity of JNJ 39758979 for the H_4R versus the histamine H_1 receptor was confirmed in vivo in humans. This clinical study proved a role for the H_4R in histamine-induced pruritus in humans and suggests that antagonists of the receptor may be efficacious in pruritic conditions driven by histamine such as urticaria.

Atopic dermatitis is a disease where pruritus can be the most troubling symptom. However, histamine was not thought to be involved since antihistamines that target the histamine H_1 receptor are not effective in managing the pruritus or the overall disease (Thurmond et al. 2015). The fact that the H_4R is involved in mediating histamine-induced pruritus in humans provided rationale for reinvestigating the role of histamine in atopic dermatitis. In addition to this, H_4R antagonists have

shown activity against pruritus in a number of mouse preclinical models of dermatitis (Rossbach et al. 2009; Cowden et al. 2010b; Suwa et al. 2011; Ohsawa and Hirasawa 2012). Further rationale was provided by the efficacy of H_4R antagonists on inflammatory parameters in these mouse models. In particular, JNJ 7777120, JNJ 28307474, and JNJ 39758979 have all been shown to reduce inflammation in an FITC-mediated model that has a phenotype similar to atopic dermatitis in that it is Th2 driven and leads to the accumulation of eosinophils and mast cells at the site of inflammation (Cowden et al. 2010b; Thurmond et al. 2014). H_4R-deficient mice also had a reduction in inflammation in this model similar to what was seen with the antagonists (Cowden et al. 2010b). Efficacy with H_4R antagonist has also been observed in other chronic allergic dermatitis models (Seike et al. 2010; Matsushita et al. 2012; Ohsawa and Hirasawa 2012; Mahapatra et al. 2014).

With this rationale, a clinical study was conducted to test efficacy of JNJ 39758979 in atopic dermatitis patients (Murata et al. 2015). Two dose levels of JNJ 39758979, 100 and 300 mg/day, were compared to placebo. These doses were selected because they were the highest tolerated doses in the phase 1 studies and provided through exposures well above those needed for efficacy in mouse models, 170 nM (38 ng/mL). The study was terminated early due to safety reasons (see below), and thus only 50 of the planned 105 patients reached the primary endpoint at 6 weeks. Nevertheless, the post-hoc results indicated that JNJ 39758979 appeared to have efficacy in atopic dermatitis. The primary endpoint for the study was a change in the Eczema Area and Severity Index (EASI) (Hanifin et al. 2001) compared to placebo. This index measures several parameters related to the skin lesions associated with atopic dermatitis including erythema, infiltration/population, excoriation, and lichenification. Both the 100 and 300 mg arms of the study exhibited numerical improvements in EASI compared to placebo starting at week 1; however, the results were not statistically significant with the caveat that the sample size was very small. These results suggest that H_4R antagonists can be effective in treating atopic dermatitis. As confirmation of the results seen with JNJ 39758979, another H_4R antagonist, ZPL-389, was recently shown to also be effective in patients with atopic dermatitis. In this case the reductions in EASI compared to placebo were nominally statistically significant (Werfel et al. 2016).

As mentioned above, itch is one of the characteristic symptoms of atopic dermatitis (Williams 2005). JNJ 39758979 was able to reduce pruritus in atopic dermatitis patients in the clinical study (Murata et al. 2015). There is no standard way to assess pruritus in the clinic, however, one of the most commonly used methods is a numerical rating scale where patients are asked to rate their itch on 0–10 scale. In the clinical study with JNJ 39758979 this was administered by electronic devices twice a day (morning for pruritus overnight and evening for pruritus during the day) for both the severity and duration of itch. For both of these parameters, patients taking JNJ 39758979 reported lower scores than those on placebo and the results reached nominal statistical significance for the 300 mg group. The improvement in the severity of itch was of similar magnitude to that seen with dulipumab (Thaci et al. 2015). Similar effects were seen by using other daily tools including a pruritus categorical response scale and a pruritus

interference numeric rating scale. Even more impressive were results obtained when a subject's global impression of change was used. In this case at each visit (roughly every 1–2 weeks) subjects were asked to rate their pruritus intensity and duration compared to the beginning of the study. At week 6 between 70 and 90% of the patients on JNJ 39758979 reported that the intensity was less and the duration of itch was shorter compared to what was observed at the beginning of the study, whereas only 30–40% of the patients on placebo reported improvements in these parameters. All of these results were nominally statistically significant.

Of potential interest is that the time course for improvement of EASI is similar to that of pruritus. One hypothesis is that H_4R antagonists would directly block the transmission of the pruritic signal. This is most likely the case for histamine-induced itch. If this was true for atopic dermatitis, then one would predict that the onset of pruritus relief would be rapid. However, it appears that it takes several weeks and parallels the improvement in inflammation. This suggests that the mechanism for the anti-pruritic effect of H_4R antagonists in atopic dermatitis may be a result of the anti-inflammatory effects and not a direct effect on pruritic signals. Overall, the pruritus results combined with the effects on the skin lesions suggest that H_4R antagonists may be promising future drugs for the treatment of atopic dermatitis.

While other H_4R antagonists may eventually be available for the treatment of atopic dermatitis, the development of JNJ 39758979 was terminated. In the atopic dermatitis study two patients receiving the 300 mg dose of JNJ 39758979 developed agranulocytosis (Murata et al. 2015). Agranulocytosis is a severe form of neutropenia where the absolute neutrophil count drops to less than 0.5×10^9/L. Fortunately, both patients recovered once they stopped taking JNJ 39758979. Drug-induced agranulocytosis is a rare, idiosyncratic disorder that has been reported for a number of different drugs (Andersohn et al. 2007). The reduction in neutrophils is thought to be the result of either apoptosis of neutrophils themselves, activation of immune mechanisms to target neutrophils, or disruption of myelopoiesis. While the exact mechanisms leading to these effects are unknown, the current hypothesis is that reactive intermediate(s) of the drug play an important role in the pathogenesis (Tesfa et al. 2009). These reactive intermediates can be formed via the normal metabolic pathways for the drug or, as is the putative case for clozapine, the generation of reactive intermediate(s) can result from reactions with compounds produced by activated neutrophils. Therefore, the most likely cause for the agranulocytosis seen with JNJ 39758979 is the formation of reactive intermediate(s). One reactive metabolite of JNJ 39758979 has been identified, but the actual identity of the species leading to agranulocytosis is unknown and may only be present in tissues (Murata et al. 2015).

Agranulocytosis could also occur by mechanism-based disruption of myelopoiesis. One report has shown the H_4R is expressed on murine and human progenitor cells and in vitro data from this paper indicate that agonists of the receptor reduce growth factor-induced cell cycle progression that leads to decreased myeloid, erythroid, and lymphoid colony formation (Petit-Bertron et al. 2009). To determine if JNJ 39758979 had such an effect, the impact of JNJ 39758979 on

human myeloid colony formation was studied in vitro (Thurmond and Dunford, unpublished data). Briefly, human bone marrow derived hematopoietic progenitor cells were cultured in methylcellulose-based media containing the appropriate recombinant cytokines to differentiate the stem cells to myeloid colony forming units and their granulocyte or macrophage sub-sets. Mature hematopoietic colonies were assessed and scored. No effects on human myelopoiesis were detected with JNJ 39758979 in vitro up to a highest concentration of 30 μM. In addition such effects on myelopoiesis should be detectable in preclinical toxicology studies unless they were human specific. However, neutrophil levels and neutrophil turnover were normal in H_4R-deficient mice and in rat and monkey toxicity studies, as well as there being no signs of bone marrow abnormalities or toxicity. These results support the conclusion that the compound is unlikely to have any direct inhibitory effects on myelopoiesis and the most likely explanation for the agranulocytosis observed with JNJ 39758979 is the formation of reactive species, consistent with the current thinking of the mechanisms by which other drugs cause agranulocytosis.

4 The Development of Toreforant

Since the formation of reactive intermediates is related to the structure of the compound, the best way to mitigate this would be to develop compounds with structural differences that are metabolized differently. The best example of this is clozapine that has a warning in its label for the occurrence of agranulocytosis with an estimated yearly incidence rate of 1.3%. However, the drug olanzapine, which is closely related structurally, causes little, if any, agranulocytosis (Naumann et al. 1999). Fortunately, a second H_4R antagonist, toreforant (pronunciation – tor ef' oh rant), was being developed in parallel. This molecule was structurally distinct from JNJ 39758979 (Fig. 1). This compound is derived from the pyridinyl benzimidazole series represented by JNJ28307474, with key modifications leading to the replacement of the core pyridine with an aminopyrimidine. Overall the pharmacology of toreforant was similar to JNJ 39758979 and previous H_4R antagonists (Table 2). One notable exception is that the affinity for the mouse H_4R is reduced compared to JNJ 7777120 and JNJ 39758979 and thus high doses are needed for efficacy in mouse disease models. That being said, the compound has efficacy in mouse models of asthma, dermatitis, and arthritis similar to that of JNJ 39758979 (Table 3) (Thurmond et al. 2017). However, one difference is that toreforant had no activity against histamine-induced scratching in mice, even at very high doses (Thurmond et al. 2017). This may be due to the fact that toreforant does not cross the blood–brain barrier, due to being a substrate for P-glycoprotein, and central nervous system activity may be required to block histamine-induced itch in mice.

JNJ 7777120 has been shown to have activity in a variety of pain models including models of inflammatory pain, neuropathic pain, pain associated with osteoarthritis, and post-operative pain and this may be related to similar mechanisms that mediate the anti-pruritic effects (Coruzzi et al. 2007; Altenbach et al. 2008; Cowart et al. 2008; Hsieh et al. 2010). Both JNJ 39758979 and toreforant

have been tested in a rat spinal nerve ligation model. At 50 mg/kg p.o. JNJ 39758979 yielded a 50% reduction in pain responses (Thurmond et al. 2017). Efficacy was also been seen in a rat mild thermal injury model. In contrast toreforant did not show efficacy in either model despite having more affinity for the rat H_4R than JNJ 39758979 (Thurmond et al. 2017). As with the pruritus model this could be related to the lack of CNS penetration for toreforant. However, it should be noted that the presence of the H_4R in the brain is controversial (Panula et al. 2015; Schneider and Seifert 2016).

While histamine has mainly been associated with allergic, Th2-type conditions, there is emerging evidence that it, and the cells that produce it, may be important in autoimmune diseases like rheumatoid arthritis (RA) (Zhang et al. 2006). Increases in histamine levels have been reported in the plasma and synovial fluid of RA patients and, in addition, mast cells appear to be increased in synovial fluid (Crisp 1984; Godfrey et al. 1984; Frewin et al. 1986; Tetlow and Woolley 1995, 1996; Gotis-Graham and McNeil 1997; Gotis-Graham et al. 1998). The H_4R appears to be expressed in synovial cells from subjects with RA (Ikawa et al. 2005). Preclinically H_4R-deficient mice or mice treated with H_4R antagonists showed reduced symptoms and inflammation in models of RA (Cowden et al. 2013; Savall et al. 2014). Toreforant shows a similar effect in reducing disease score in a mouse model of arthritis (Thurmond et al. 2017).

With this rationale in hand, two studies with toreforant were conducted in patients with RA (Thurmond et al. 2016). The first study was a phase 2a study comparing 100 mg/day toreforant to placebo in RA patients on stable doses of methotrexate who still exhibited disease activity. This study was planned to enroll approximately 90 subjects (2:1 active:placebo randomization) and for them to be treated for 12 weeks. However, the study was terminated early due to what at the time was an unexplained death in a patient who received toreforant. It was later determined that the cause of death was secondary hemophagocytic lymphohistiocytosis, an immune activation syndrome, and unlikely to be related to toreforant, although the relation cannot be ruled out entirely. Due to the early termination, only 36 subjects completed the study and the efficacy analysis was post-hoc. Nevertheless, it appeared that subjects taking toreforant had improvements in signs and symptoms associated with RA (Thurmond et al. 2016). A follow-up study was conducted in the same patient population, but this time assessing 3, 10, and 30 mg/day doses of toreforant over 6 months (Thurmond et al. 2016). The top dose of toreforant was lowered compared to the phase 2a study based on preclinical efficacy models predicting that trough values of 6 ng/mL would be necessary for efficacy and the 30 mg dose yields a trough value significantly above this, 104 ng/mL. In addition, QT prolongation was observed in subjects at doses above 100 mg, making long-term development of the 100 mg dose problematic (Thurmond et al. 2017). In contrast to what was seen in the phase 2a study, no efficacy was observed for toreforant on any efficacy parameter in RA patients (Thurmond et al. 2016). Given the disparity between the results it is unclear as to whether the lack of efficacy in the second study was due to the lower doses used or misleading results from the first study due to the early termination. Overall, it is still unclear whether H_4R antagonists will have efficacy in RA.

5 Concluding Remarks

In conclusion, the first reports of clinical data with H_4R antagonists have appeared over the past couple of years; however, as evidenced by the history with the Janssen H_4R program, the path to the clinic has not been a smooth one. In the process of developing compounds that target the H_4R, thousands of compounds have been made that are ligands for the receptor; however, very few of them have all of the properties needed to advance into clinical testing. This is not unique for the H_4R, as finding ligands and inhibitors for drug targets is just a starting point and the real difficulty lies in finding a compound with the right properties to make it a useful therapeutic. Fortunately for the H_4R, compounds like JNJ 39758979 and toreforant exhibited profiles that allowed clinical testing and so the role of the H_4R in human diseases can be explored. Clinical work with these compounds have shown the clear potential for the use of H_4R antagonists in the treatment of pruritus and atopic dermatitis and it may not be long before drugs that target the H_4R are available for the treatment of such conditions. However, the efficacy in rheumatoid arthritis is still not clear and results from other studies conducted in allergen challenge models, asthma, and psoriasis have yet to be reported. In conclusion, there has been much progress in identifying useful ligands for the H_4R, understanding its role in preclinical models and early success in clinical studies. However, the next frontier will be to detail out the full therapeutic potential of H_4R antagonists and the full spectrum of human diseases where the H_4R plays a role.

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Allergy, Histamine and Antihistamines

Martin K. Church

Contents

Abstract

This chapter concentrates on the role in allergic disease of histamine acting on H_1-receptors. It is clear that allergy has its roots in the primary parasite rejection response in which mast cell-derived histamine creates an immediate hostile environment and eosinophils are recruited for killing. This pattern is seen in allergic rhinitis where the early events of mucus production and nasal itching are primarily histamine mediated whereas nasal blockage is secondary to eosinophil infiltration and activation. In asthma, the role of histamine is less clear. Urticaria is characterized by mast cell driven pruritic wheal and flare-type skin reactions that usually persist for less than 24 h. Although the events leading to mast cell degranulation have been unclear for many years, it is now becoming evident that urticaria has an autoimmune basis. Finally, the properties of first- and second-generation H_1-antihistamines and their role in allergic is discussed.

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Allergy • Asthma • H_1-antihistamines • Parasitology allergic rhinitis • Urticaria

1 Introduction

While this chapter concentrates on the role in allergic disease of histamine acting on H_1-receptors, histamine may also act at three other receptor subtypes. Of these, the H_4-histamine receptor is probably most relevant to allergy (Thurmond 2015). The ability of H_4-antihistamines to reduce allergic inflammation and eosinophil accumulation in asthma (Dunford et al. 2006) and to reduce itching in skin diseases (Thurmond 2015) suggests that their development open the door to new treatments for allergic disease. The roles of the H_4-receptor in asthma and pruritic skin disease are addressed by Detlef Neumann and Rob Thurmond, respectively, in Chaps. 17 and 18.

2 The Roots of Allergy

The immune system has diverse ways of dealing with invading organisms. Invaders that have defeated the external defence line and have entered the body may be dealt with by one of the two systems, the innate immune system, which is nonspecific and does not require previous exposure and does not improve with repeated exposure to infection, and the acquired immune system which requires previous exposure and is highly specific for a particular invader. With small invaders, such as viruses and bacteria, their recognition by a variety of mechanisms is usually followed engulfment and intracellular digestion by macrophages. However, such a mechanism is not possible with larger invaders such as helminth parasites. For such invaders, the immune system uses a completely different approach. Initial infestation leads to a Th2 immune response and the production of IgE specific to parasite antigens (Murphy et al. 2008). This IgE binds to high affinity (FcεRI) receptors on mast and basophils thus arming them for any subsequent attack. On such a second attack, recognition by IgE of the parasite antigen initiates primary and secondary events. The primary event is IgE-dependent mast cell degranulation resulting the release into the extracellular environment of histamine, heparin and neutral proteases (Murphy et al. 2008). The role of histamine in particular is to make the local environment hostile for the invader by increasing mucus secretion and causing sensory nerve stimulation. The latter will induce scratching, sneezing, coughing or diarrhoea depending on the organ. The secondary event is to activate the Th2 system to stimulate eosinophil influx. Eosinophils contain a spectrum of highly toxic proteins, such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil derived neurotoxin (EDN), which attack and kill the invader (Kay 1985; Puxeddu et al. 2003). In addition, both mast cells and eosinophils synthesize and secrete leukotriene C_4 (LTC_4) to increase the

sequestration of eosinophils into the local environment and enhance the killing response (Kay 1985).

However, sometimes the immune system gets it wrong! It has long been recognized that the genetic make-up of some individuals renders them atopic or susceptible to allergic disease (Holloway et al. 2010b). In atopic individuals, allergens, such as house dust mite, tree and grass pollen and fungal spores, are mistakenly recognized as nematode antigens and they mount an allergic attack, which, to all intents and purposes, is identical to a parasite rejection response.

3 Allergic Rhinoconjunctivitis

Perhaps the easiest allergic response to address is allergic rhinitis. The early phase of this is shown diagrammatically in Fig. 1 (Church et al. 2016). In the upper panel of this figure, allergen, such as pollen or house dust mite, penetrates the nasal epithelium (A). This allergen then interacts with IgE (B) to stimulate mast cell degranulation and the release of its preformed mediators including histamine (C). In this early phase response, histamine has three immediate effects: first, it stimulates mucosal goblet cells to produce watery mucus (D); second, it stimulates sensory nerves to cause nasal itching and sneezing (E) and third, it induces vasodilation and tissue oedema which contribute to nasal blockage (F). Because these effects are primarily histamine mediated, H_1-antihistamines are effective in relieving these symptoms.

This early phase response is followed by the development of allergic inflammation that increases the severity and persistence of the initial symptoms, resulting in a chronic phase of allergic rhinitis. This is shown in the lower panel of Fig. 1. Cytokine production by mast cells and Th2 lymphocytes causes the attraction of more mast cells and the influx and activation of other inflammatory cells, particularly eosinophils (G) (Westergren et al. 2009). Eosinophils contain aggressive proteinaceous mediators as described above (H) that stimulate sensory neurones to dramatically increase the production and release of neuropeptides (I). These neuropeptides act on special venous capacitance vessels (J) present in the nasal turbinates, causing dilatation and reduced emptying. This is the primary cause of nasal blockage. Although these effects may be reduced by the inflammatory actions of H_1-antihistamines (Patou et al. 2006), intranasal corticosteroids or leukotriene receptor antagonists are more effective (Scadding et al. 2008).

Allergic rhinitis is often accompanied by ocular symptoms, particularly reddened, itchy and watery eyes. Originally it was believed that these conjunctival symptoms were caused by the activation of conjunctival mast cells with airborne allergen (Baroody et al. 2008). However, it is now believed that these symptoms are mainly the result of a naso-ocular reflex in which allergic inflammation in the nose stimulates the trigeminal nerve with subsequent release of neuropeptides in the tears (Callebaut et al. 2012). These neuropeptides activate conjunctival mast cells to release histamine but little cytokine. Consequently, there is little subsequent eosinophil infiltration and allergic inflammation in mild allergic

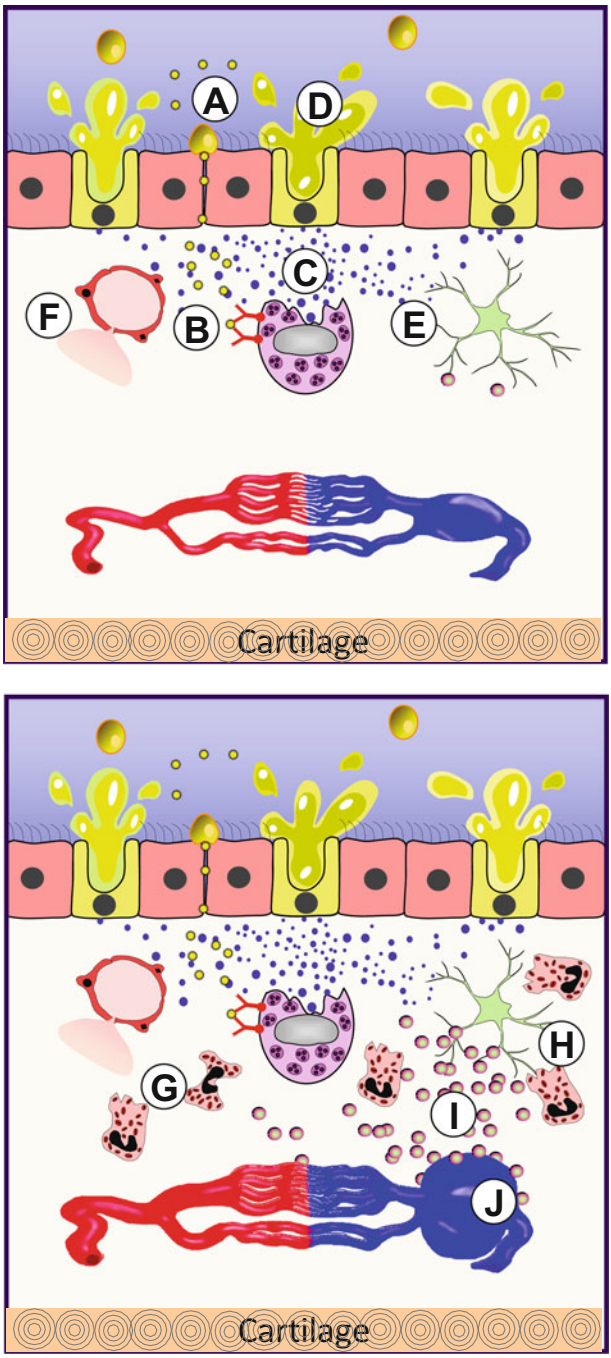

Fig. 1 The cellular basis of allergic rhinitis. The early phase is shown in the *upper panel* and allergic inflammation and nasal blockage in the *lower panel*. The *letters* are explained in the text. Adapted from Church et al. (2016)

rhinoconjunctivitis (Baroody et al. 2008). Rhinoconjunctivitis may be treated by reducing nasal inflammation, and hence the nasal reflex, with intranasal steroids, by the use of H_1-antihistamine eye drops or by topical chromones to reduce mast cell degranulation and sensory nerve activation (Baroody and Naclerio 2011).

A more severe form of allergic conjunctivitis is vernal conjunctivitis (McGill et al. 1998). This potentially sight threatening condition occurs in areas of high atmospheric pollen levels, such as the Middle East Asia, Africa and Mediterranean areas, where pollen impacts directly on the conjunctiva to initiate an allergic response. Because this is a direct allergen-induced response, complete activation of mast cells occurs (Church and McGill 2002) with the resultant cytokine production stimulating eosinophil infiltration and allergic inflammation (McGill et al. 1998).

4 Asthma

Some 30 years ago, asthma was considered to be primarily a mast cell mediated disease. Indeed, in his review of the pathophysiology of asthma in 1979, James Hogg wrote (Hogg et al. 1979) 'the initial event in an acute asthmatic attack is the release of mediators from superficial mast cells, and this amplifies the allergic response by altering the mucosal permeability so that more antigen reaches the sub-mucosal mast cells. This altered permeability may also help explain the hyper-reactivity of the airways to nonspecific airway stimulants in persons with asthma'. In line with this view, acute anaphylactic bronchoconstriction in guinea pigs (Kallos and Kallos 1984) and rats (Church et al. 1972) were used as test models in the quest to search for drugs for the treatment of asthma. No effective drugs were found. Today our view of asthma is completely different. Asthma is now viewed as a multifactorial chronic inflammatory condition whose disease progression is under the influence of a wide variety of genes which are associated with many aspects of the condition, altered lung development, response to the environment, fixed airway obstruction and response to therapy (Holloway et al. 2010a). What is most fascinating is that susceptibility to atopy/allergy is now relegated to the level of one of the contributory factors.

However, when discussing allergic asthma in particular, we cannot ignore the fact that 'the nose is the guardian of the lung' and that histamine may be involved here. In a study published in 2004, Corren and colleagues (Corren et al. 2004) investigated whether treatment with intranasal corticosteroids and/or second-generation H_1-antihistamines (SGAHs) was associated with changes in rates of asthma exacerbations resulting in emergency room visits and/or hospitalizations in patients with asthma and allergic rhinitis. Their results showed that treatment with either nasal corticosteroids or SGAHs was associated with a lower risk of asthma-related emergency room treatment and hospitalization. Combined treatment with both medications was associated with a better effect of either alone.

5 Urticaria

Urticaria is one of the most frequent skin diseases. It is characterized by mast cell driven pruritic wheal and flare-type skin reactions that usually persist for less than 24 h. Some patients have both wheals and angio-oedema, while others have only one or the other, usually the former. According to the current EAACI/GA2LEN/EDF/WAO guideline, urticaria may be divided into spontaneous urticaria, physical urticaria and other urticaria types (Zuberbier et al. 2009). Spontaneous urticaria is further divided into acute and chronic spontaneous urticaria (CSU) depending on whether the disease duration is less than or more than 6 weeks. The triggers of the physical and other urticaria types, e.g. cold, heat, scratching, pressure or exercise, are obvious. In contrast, in spontaneous urticaria the lesions usually occur without an obvious stimulus, although foods, infections and stress have been suggested as possible stimuli (Maurer et al. 2011b).

While the role of mast cell histamine in the pathogenesis of all forms of urticaria is clear from the beneficial effects of H_1-antihistamines, the mechanisms by which the mast cell is activated is far from clear. However, the recent reports that anti-IgE (omalizumab) given by subcutaneous injection at monthly intervals is highly effective in relieving the symptoms of CSU in the majority of patients (Maurer et al. 2015) is suggestive of a critical role of IgE in this condition. Because some patients respond to anti-IgE rapidly, within 1 week, while others may take up to 2 months to respond (Metz et al. 2014), we have recently postulated two possible types of Gell and Coombs hypersensitivity mechanism reactions (Coombs and Gell 1968; Kolkhir et al. 2016).

The rapid response we suggest is a Type I hypersensitivity, or allergic reaction, in which free antigens cross-link the IgE on mast cells and basophils to cause the release of vasoactive mediators (Fig. 2). This was first suggested by Rorsman in 1962 to explain urticaria associated basopenia (Rorsman 1962). That this was an autoallergic response that was postulated from the finding in 1999 of IgE autoantibodies against the thyroid microsomal antigen in the serum of a female patient who suffered from CSU (Bar-Sela et al. 1999). This work has been confirmed and extended to propose autoallergy in the pathogenesis of both CSU and chronic inducible urticaria (CindU) (Concha et al. 2004; Kaplan 2004; Altrichter et al. 2011; Maurer et al. 2011a; Shindo et al. 2012; Hatada et al. 2013).

A Type II hypersensitivity reaction in which antibodies, usually IgG or IgM, bind to antigen on a target cell was originally postulated following the identification in three of six patients with CSU of IgG antibodies against IgE which caused degranulation of leukocytes (Fig. 2) (Gruber et al. 1988). The presence of these antibodies was confirmed by Grattan and co-workers in 1991 in patients whose sera induced a wheal and flare response when injected intradermally, the autologous serum skin test (ASST) (Grattan et al. 1991). The presence of antibodies to the high affinity receptor for IgE on mast cells and basophils (IgG anti-FcεRI) was reported by the same group 2 years later (Hide et al. 1993). This has now been confirmed by many authors (Kaplan 2004; Kaplan and Greaves 2009).

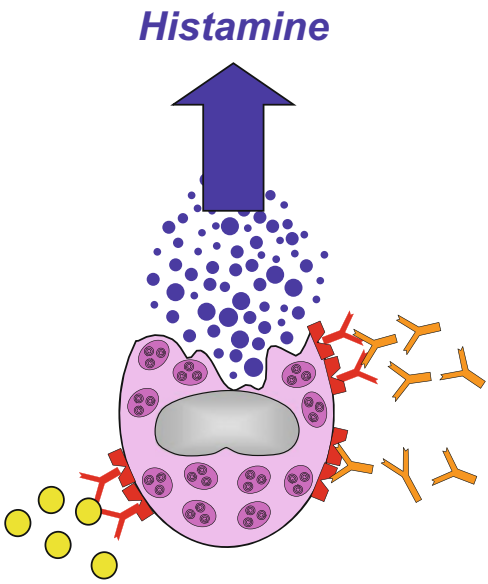

Fig. 2 Possible autoallergic mechanisms of chronic spontaneous urticaria (CSU). On the *left*, autoallergen cross-links membrane-bound IgE. On the *right*, IgG antibodies are cross-linking IgE or their receptors, FcεRI

Thus, there are still many aspects of the pathologic mechanisms of CSU that need to be resolved, but it is becoming clear that there are at least two distinct pathways, type I and type II autoimmunity, that contribute to the pathogenesis of this complex disease (Kolkhir et al. 2016).

6 H_1-Antihistamines

Oral H_1-antihistamines are the first-line treatment used by most patients, doctors and pharmacists for allergic rhinoconjunctivitis and urticaria. Histamine acting on H_1-receptors in the brain has a completely different function to that in the periphery. In the brain, it has an arousal effect and aids concentration and learning (Church et al. 2010). Thus, when selecting an H_1-antihistamine for treatment, healthcare professionals should be aware of the significant detrimental effect of agents that cross the blood–brain barrier to cause effects on the central nervous system (CNS). First-generation H_1-antihistamines (FGAHs) such as chlorpheniramine, diphenhydramine, hydroxyzine and ketotifen readily penetrate into the brain and occupy more than 50% of the H_1-receptors therein (Yanai et al. 2011). Occupation of these receptors results in drowsiness and interference with cognitive processes in all patients (Church et al. 2010).

Studies in children have demonstrated that FGAHs exacerbate the detrimental effect of allergic rhinitis on learning ability (Vuurman et al. 1993). In another study in teenagers sitting summer mock university entrance examinations, untreated allergic rhinitis caused a 40% increased likelihood of students dropping an examination grade. FGAHs increased this to 70% (Walker et al. 2007).

In adults, FGAHs have detrimental effects on the quality of life and productivity even at the lowest doses recommended by manufacturers (Church et al. 2010). The effects of FGAHs on the CNS are similar to and additive with those produced by alcohol or other CNS sedatives, and bedtime dosing with FGAHs may have hangover effects the next day due to their long elimination half-life value (Church et al. 2010). Furthermore, FGAHs may significantly reduce the driving ability to potentially dangerous levels (Verster and Volkerts 2004; Church et al. 2010), particularly in the elderly and those who combine the drug with alcohol ingestion. One study suggests that 25% of individuals older than 65 years of age have some cognitive impairment, often with no overt sign of dysfunction (Graham et al. 1997). Administration of FGAHs to this population increases the risk of inattention, disorganized speech, altered consciousness and impaired function or alertness (Agostini et al. 2001; McEvoy et al. 2006). In addition, because of their anticholinergic activity, FGAHs significantly increase the risk for development of dementia (Gray et al. 2015).

The development of SGAHs, including loratadine, desloratadine, cetirizine, levocetirizine and ebastine, has largely overcome these problems in that they have high H_1-receptor selectivity, low brain permeability and longer durations of action. Unlike FGAHs, SGAHs are amphiphilic in that hydrophilic groups have been introduced into the molecule so that they are always positively or negatively charged and, therefore, have a greatly reduced passage across the blood–brain barrier (Yanai et al. 2011; Hiraoka et al. 2015). Because of their reduced unwanted effects, the European Guidelines for both allergic rhinitis and urticaria specify that only SGAHs should be used for symptom relief in these conditions (Scadding et al. 2008; Zuberbier et al. 2009). Although SGAHs have a much reduced brain penetration, they may only be referred to as 'minimally sedating' rather than 'non-sedating'. For example, in a study of patients' perspective of effectiveness and side effects of H_1-antihistamine updosing in CSU, more than 20% of patients reported sedation is a side effect of SGAHs (Weller et al. 2011).

More recently, two truly 'non-sedating' H_1-antihistamines, fexofenadine and bilastine, have been introduced which have no significant occupation of histamine H_1-receptors in the CNS (Hiraoka et al. 2015; Farre et al. 2014). The reason for their lack of brain penetration is that they are actively pumped out of the blood–brain barrier by p-glycoprotein (a proton pump) (Miura and Uno 2010; Church 2011). It will be interesting to see if further drugs will be developed which use membrane proton pumps to enhance their efficacy or reduce their unwanted effects.

7 Conclusions

While it is clear that histamine acting at H_1-receptors are critically involved in producing rhinorrhea, itching and sneezing, it is equally clear that it is not involved in nasal obstruction. In asthma too, histamine has only a minor role. In chronic urticaria, the application of guideline-based management following specialist review, i.e. updosing H_1-antihistamines fourfold, was associated with a good

outcome in only 78% of patients (Conlon and Edgar 2014). Whether this means that mediators other than histamine are involved or that the level of histamine is extremely high in some patients is yet to be clarified.

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Histamine and Histamine Receptors in Allergic Dermatitis

Hiroshi Ohtsu and Masahiro Seike

Contents

Abstract

In this chapter we will first introduce the pathophysiological process of several skin diseases including allergic dermatitis, a common skin disease, including chronic allergic contact dermatitis (CACD), and atopic dermatitis (AD). In CACD and AD patients, repeated skin exposure to antigens contributes to the development of chronic eczematous lesions. Repeated application of haptens on mice allows emulation of the development of CACD in humans. Further, we will focus on H1, H2, and H4 histamine receptors and their effects on CACD and AD. Histamine-deficient mice, with a knockout histidine decarboxylase (HDC)

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gene, were used to investigate the role of histamine in CACD and AD. Histamine induces infiltration of inflammatory cells, including mast cells and eosinophils, and elevates Th2 cytokine levels in CACD. Histamine promotes the development of eczematous lesions, elevates IgE serum levels, and induces scratching behavior in CACD. The administration of H1 or H4 receptor antagonists was effective to ameliorate these symptoms in murine CACD models. The combination of H1 and H4 receptor antagonists is a potential therapeutic target for chronic inflammatory skin diseases such as CACD and AD, since combined therapy proved to be more effective than monotherapy.

Keywords
Allergic dermatitis • Atopic dermatitis • Chronic allergic contact dermatitis • Histidine decarboxylase (−/−) mice

1 Introduction

Dermatitis is the most common disease in dermatology practice. Dermatitis is characterized by itching and soreness of variable intensity, and in variable degrees, a range of signs, including dryness, erythema, excoriation, exudation, fissuring, hyperkeratosis, lichenification, scaling, and vesiculation. Dermatitis can be called acute, subacute, or chronic, depending on its clinical and pathological features. Besides this classification, there is no international agreement on the subcategories of dermatitis; only dermatitis with specific names according to their distinguishing features is clinically considered.

Contact dermatitis is known as one of the most common types of dermatitis, with itch sensation (Thomson et al. 2002). Three types of contact dermatitis are known: (a) irritant contact dermatitis, (b) allergic contact dermatitis (ACD), and (c) photocontact dermatitis. (a) Irritant contact dermatitis is triggered by chemical or physical irritant factors. Chemical irritants including solvents, surfactant, acid, and alkalis are able to induce contact dermatitis. The most common physical irritant is low humidity due to air-conditioning (Morris-Jones et al. 2002). (b) ACD is clinically problematic, since it is a very common occupational- and environmental-related disease. Together with other forms of allergies, the ACD progresses in two stages: an initial sensitization phase, followed by an elicitation phase (Kimber et al. 2002). In metal allergy, one of the most representative forms of ACD, allergens include nickel, chromium, and cobalt. Phototoxic contact dermatitis is more common than photoallergic contact dermatitis and resembles severe sunburn. Photoallergic contact dermatitis or photocontact dermatitis resembles ACD on sun-exposed areas, although sometimes it may extend to covered areas as well (Lugović et al. 2007; Honari 2014).

Atopic dermatitis (AD) is a common, chronic or chronically relapsing, severely pruritic, eczematous skin disease. The incidence of AD is considered to be increasing worldwide (Williams et al. 1999), both the adolescent and adult types of AD

(Takeuchi and Ueda 2000). AD is a chronic inflammatory skin disease characterized by eczematous skin lesions and intense pruritus (Kabashima 2013). The immunological Th2 response has been found to play a key role in the pathogenesis of AD (Bieber 2010). The production of Th2 cytokines, namely, interleukin (IL)-4, IL-5, and IL-13, increases immunoglobulin (Ig) E production and/or eosinophil activation, subsequently amplifying the allergic inflammation (Brandt and Sivaprasad 2011; Guttman-Yassky et al. 2011). Levels of total IgE (Gebhardt et al. 1997) and peripheral eosinophil counts (Simon et al. 2004) correlate with AD severity.

In this chapter we will focus on the role of histamine in a murine AD model, especially in relation to T-helper cells, the activity of histamine on the scratching behavior, and subtypes of histamine receptors with type-specific activity.

2 Allergic Dermatitis

Allergic dermatitis includes ACD and AD. ACD basically occurs as a type IV allergic reaction. The causative agent invades the body percutaneously and is captured by epidermal antigen presenting cells, i.e., Langerhans cells. These cells migrate to the regional lymph nodes and induce T cell activation. Consequently, T cells proliferate in the lymph nodes achieving sensitization. When the same agent reinvades the skin, the sensitized T cells activate and release inflammatory cytokines leading to dermatitis. Among numerous different inflammatory cells involved in the pathogenesis of dermatitis, including antigen presenting cells, granulocytes, and keratinocytes, T cells play a pivotal role among immune effectors in allergic dermatitis (Novak et al. 2003). AD is defined as a disease in which the main lesion is an itching eczema with recurrent remissions and exacerbations. Most patients also show an atopic condition (Saeki et al. 2009). In most AD cases, in addition to type IV allergic reactions, type I allergic reactions (e.g., atopic conditions such as urticaria, allergic rhinitis, and asthma) are also involved.

A widely used mouse model of ACD is the delayed-type hypersensitivity response (type IV allergic reaction) to small organic haptens with potent sensitizing capacity (Grabbe and Schwarz 1998). As previously discussed, AD is predominantly orchestrated by Th2 cells, while ACD is considered to be a Th1 dominant disease. In patients with chronic allergic contact dermatitis (CACD), the repeated exposure to antigens through the skin is thought to contribute to the development of the eczematous lesions. In the murine model, repeated applications of an antigen result in antigen-specific hypersensitivity responses. The skin reaction in mice changes from a delayed- to an early-type response in correlation to the increased number of repeated applications of the allergen, and finally accompanied by the accumulation of mast cells in the upper part of the dermis and elevation of serum IgE levels (Kitagaki et al. 1995).

AD, aggravated by chronic exposure to antigens, is a common and distinctive form of allergic skin diseases associated to eczematous lesions, early-type hypersensitivity responses, and increased IgE production in response to environmental

allergens (Cooper 1994; Ohmen et al. 1995). AD bears clinical, histological, and immunological similarities to CACD (Wang et al. 2007; Man et al. 2008). Both CACD and AD are Th2 dominant diseases.

3 The Role of Histamine in Atopic Dermatitis

3.1 Pathogenetic Role of Histamine

The pharmacological assessment of the in vivo effects of histamine in the development of eczematous lesions was an issue prior to the development of histidine decarboxylase (HDC) knockout mice, since most observations involve the use of histamine receptor antagonists. To overcome this limitation, we produced histamine-deficient mice by disrupting the HDC gene (Ohtsu et al. 2001).

First, we observed a skin allergic reaction. Plasma extravasation after a challenge with compound 48/80, a compound which induces an immediate-type allergic response, was positive in HDC (+/+) mice but negative in HDC (−/−) mice (Ohtsu et al. 2002). On the other hand, contact hypersensitivity, a delayed-type allergic response, showed no difference in a model of thickening of the ear skin after trinitrochlorobenzene (TNCB) between HDC (+/+) and HDC (−/−) mice (Ohtsu et al. 2002; Seike et al. 2010).

A repeated epicutaneous application of sensitizing agents develops a skin reaction characterized by epidermal hyperplasia, accumulation of large numbers of mast cells and CD4+ T cells beneath the epidermis, and increase in the serum levels of antigen-specific IgE (Kitagaki et al. 1995), similar to the observations in AD (Kim et al. 2015). The contact hypersensitivity response shifts from a delayed-type hypersensitivity to an immediate-type response due to repeated applications (Kitagaki et al. 1995). The role of histamine in the extent of the skin reaction induced by repeated applications of contact sensitizing agents was investigated on HDC (−/−) mice. Histological examination of the skin reveals that mice display hyperplastic epidermis and infiltration of mast cells, CD4+ T cells, and eosinophils following repeated daily applications of diphenylcyclopropenone (DCP) to the skin. The magnitude of these changes was more significant in HDC (+/+) than in HDC (−/−) mice (Seike et al. 2005a, 2010). This finding suggests that histamine promotes the development of chronic eczematous-like lesions.

3.2 Histamine and Th2, Th1, Treg, and Th17

In a repeated challenge with TNCB, which is the model for CACD, the levels of Th2 cytokines IL-4 and IL-5 were higher in skin lesions of HDC (+/+) than of HDC (−/−) mice. On the other hand, IFN-γ and IL-12 levels, representative Th1 cytokines, showed no significant changes in skin lesion from HDC (+/+) or HDC (−/−) mice. Serum IgE levels in HDC (+/+) mice were higher than in HDC (−/−) mice (Seike et al. 2010). From these observations, together with another report

(Mahapatra et al. 2014), we can conclude that histamine seems to induce Th2 dominant allergic reactions in CACD.

Regulatory T cells (Tregs) are a subset of T cells which regulate effector T cells and lead to immune tolerance in order to reduce allergic reactions and play a role in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes (Hori et al. 2003). Tregs play a role in ameliorating contact dermatitis by suppressing effector T cells (Ring et al. 2006). TGF-β1 is one of the main regulators of Treg recruitment in allergic lesions (Chen et al. 2003). Since the level of TGF-β1 and the number of Tregs in eczematous lesions are significantly higher in HDC (−/−) compared to HDC (+/+) mice, histamine is thought to decrease the levels of TGF-β1 and, therefore, its effect. This negative effect might help to produce the skin lesions by decreasing the number of Tregs cells (Tamaka et al. 2015).

Th17 cells, a distinct lineage of effector CD4+ T cells, are characterized by the production of IL-17 and IL-22 (Liang et al. 2006). IL-17 induces Th2 immune responses in murine AD model (Nakajima et al. 2014). Histamine might be an important regulator for Th17 recruitment in some cases, since increased Th17 levels are observed in the skin lesions in the CACD model of HDC (−/−) mice (Seike et al., unpublished data). However, in the arthritis model, it was demonstrated that the H4 receptor knockout mice and H4 receptor antagonist reduce the clinical score, which might be mediated by the reduction of IL-17 levels (Cowden et al. 2014). It was not clear whether the differential effect of histamine in CACD and arthritis is due to the differences between the models or to the specific activity of histamine and H4 receptor in allergic states. Further research is necessary to elucidate the mechanism by which histamine exerts its effect on Th17 cells.

3.3 Histamine-Induced Scratching Behavior

Pruritus has been defined as an unpleasant sensation that triggers a desire to scratch (Ikoma et al. 2011). Contact dermatitis is known as the common skin disease with itch sensation as a typical symptom (Nojima and Carstens 2003).

The role of histamine on scratching behavior and neuronal conditions has been extensively reported (Leknes et al. 2007; Nakano et al. 2008; Akiyama et al. 2009). We used HDC (+/+) and HDC (−/−) mice after daily applications of DCP to observe the long-term effects of histamine. Interestingly, scratching behavior was observed in HDC (+/+) but not in HDC (−/−) mice after DCP application (Seike et al. 2005b). A significant increase in c-Fos (+) cells was observed in lamina I in the dorsal horn of HDC (+/+) mice, whereas not in HDC (−/−) mice (Seike et al. 2005b). Therefore, the sensory cells in lamina I of the dorsal horn of HDC (+/+) mice are considered to be more excited when compared with those of HDC (−/−) mice. Moreover, substance P expression in the spinal dorsal horn has been shown to be increased with peripheral sensory stimulation after DCP treatment (Seike et al. 2005c). Since it was reported that mast cells around the nerve endings produce histamine stimulated by substance P (Erjavec et al. 1981), histamine

production might be responsible for the itch sensation in HDC (+/+) mice. E-cadherin, one of the synapse-related molecules, is expressed in the spinal dorsal horn by peripheral sensory stimulation induced in DCP-treated mice (Seike et al. 2005c). The E-cadherin expression is increased only in HDC (+/+) but not in HDC (−/−) mice. From these results we can conclude that histamine might induce E-cadherin expression either directly or indirectly. Therefore, not only the direct effect of histamine, as we will discuss further in detail, but also the indirect effect of histamine, e.g., nerve fiber proliferation and/or synapse formation, might augment the itchy sensation in this model (Seike et al. 2005b).

From these studies we concluded that scratching behavior is mainly mediated by histamine and followed by the afferent pathway of sensation connected to the central nervous system through lamina I of the spinal dorsal horn in a murine model of CACD (Seike et al. 2005b).

4 Histamine Receptors

Histamine is a ubiquitous chemical messenger, which exerts numerous functions mediated by, at least, four pharmacological distinct receptors. All histamine receptors are classified as G-coupling receptors with seven transmembrane domains (Hough 2001; Seifert et al. 2011). H1 receptors activate the PLC-IP3-Ca^{2+} pathway followed by the activation of PKC, eNOS, protein kinases, and PLA2, among other effectors. H2 receptors activate the cyclic AMP-dependent pathway, while H3 and H4 receptors activate MAP kinase, and activation of H4 receptors mobilizes Ca^{2+} ions stored inside the cells.

4.1 H1, H2, and H3 Receptors

Therefore, which kind of receptors are involved in the effect of histamine in the AD mouse model? We have previously showed that not only H1 receptors but also H4 receptors play a role in the induction of the lesion by using their specific antagonists (Seike et al. 2010; Matsushita et al. 2012). Periostin, a matricellular protein and a contributor to tissue remodeling, is a critical mediator for the amplification and persistence of allergic inflammation in a house dust mite extract-induced AD model (Masuoka et al. 2012). Histamine induces the expression of periostin in fibroblasts, and an H1 receptor antagonist blocks both periostin and collagen expression (Yang et al. 2014). Therefore, the contribution of histamine to AD through the H1 receptor might be related to periostin as well.

The activity of H2 receptor has been characterized in gastric wall cells, and its action is clinically applied for the treatment of peptic ulcer with anti-H2 blockers. In the skin, the H2 receptor is expressed in keratinocytes, macrophages, and lymphocytes (Akdis and Simons 2006). However, its function in the skin has yet not been fully understood.

H3 receptor is expressed in sympathetic and parasympathetic nerves, and it regulates the release of histamine, serotonin, acetylcholine, and other neurotransmitters (Sander et al. 2008). H3 receptor antagonists increase scratching behavior in ICR mice (Sugimoto et al. 2004) and mast cell-deficient mice (Hossen et al. 2003). Histamine induces calcium increase in skin-specific sensory neurons through the activation of the H1 and H4 receptors, as well as inhibition of the H3 receptor. The decreased threshold in the response of H3 receptor to antagonists is considered to activate H1 and H4 receptors on sensory neurons, which in turn results in the excitation of histamine-sensitive afferents and therefore elicits the itch sensation (Rossbach et al. 2011). The exact physiological role of the H3 receptor in the skin remains to be explored, although several researches suggest that H3 receptor is related to itch sensation and scratching behavior in allergic dermatitis.

4.2 H4 Receptor

Compared to the other histamine receptors, the discovery of a fourth histamine receptor was unexpected, since it was first proposed as an orphan receptor which role has been found later. H4 receptors are expressed primarily in immune cells, e.g., leukocytes and mast cells (Oda et al. 2000). In a murine Th-2 dependent skin inflammation model, H4 receptor mediates inflammation and pruritus (Cowden et al. 2010; Dunford et al. 2007; Thurmond et al. 2014). In the pruritus mouse model, the function of H4 receptor on mast cells or other hematopoietic cells seemed not to be directly related to the sensation (Dunford et al. 2007). Dunford et al. proved first that mast cell-deficient (WBBF1-W/W^v) mice showed the similar bouts of scratching behavior as the control ($WBB6F_1$-+/+) mice and second that H4 receptor knockout mice had reduced scratching bouts, which was not recovered even when the mice were reconstituted with the bone marrow cells of their wild-type counterpart. Since H4 receptors are expressed in dorsal root ganglion (DRG) neurons, the activation of afferent nerves related to the itchy sensation might be mediated by H4 type receptors (Rossbach et al. 2011).

4.3 Combined Effect of H1 and H4 Receptor Antagonists

Repeated application of haptens on the skin induces immediate hypersensitivity and produces a shift in the cutaneous cytokine milieu from Th1 to Th2 profiles (Kitagaki et al. 1997). In this model, the effects of histamine on the development of eczematous lesions were assessed using histamine-deficient mice (Seike et al. 2005a). The development of eczematous lesions in contact dermatitis was suppressed in HDC (−/−) compared to HDC (+/+) mice. Therefore histamine seems to be an important Th2 mediator in the eczematous lesion. Hence, which type of receptor plays a predominant role in these lesions?

Four types of histamine receptors have been reported to contribute to the pathophysiology of allergic dermatitis. H1 receptor antagonists inhibit murine

contact hypersensitivity but with low efficacy (Tokura et al. 2003). H2 receptor antagonists increase the contact hypersensitivity response (Belsito et al. 1990). H3 receptor antagonists have been suggested to contribute to the itchy sensation and scratching behavior, which has been already discussed in Sect. 4.1. H4 receptor antagonists reduce hapten-induced scratching behavior, but not ear swelling (Rossbach et al. 2009).

H1 receptor is definitely an important receptor in producing edema in skin diseases (Thurmond et al. 2004; Seike et al. 2005a), and these lesions were controlled, at least in part, by the effect of IL-4, IL-6, and macrophage inflammatory protein-2 (Hamada et al. 2006). On the other hand, H4 receptor by itself is a key receptor in the CACD model in mice (Seike et al. 2010). Eczematous lesions are ameliorated in the presence of H4 receptor antagonist JNJ7777120 in HDC (+/+) mice, while aggravated in the presence of H4 receptor agonist 4-methylhistamine in HDC (−/−) mice. In biochemical assays, IL-4, IL-5, and IL-6 in skin lesions and serum IgE levels are decreased, whereas IFN-γ and IL-12 levels in skin lesions are increased by the H4 receptor antagonist JNJ7777120 in this model. In histological assays, the number of mast cells and eosinophils in eczematous lesions is lower in HDC (+/+) mice after H4 receptor antagonist than in HDC (−/−) mice after H4 receptor agonist.

As previously explained, H1 and H4 receptor antagonists have beneficial effects on allergic inflammation, and the effect of the combined treatment was assessed in an AD model. The combined treatment with olopatadine and JNJ7777120, H1 and H4 receptor antagonists, respectively, reduces epidermal thickening reaction in the repeated TNCB staining CACD model (Matsushita et al. 2012). The number of eosinophils and mast cells were also decreased by the combined therapy compared to olopatadine monotherapy. Combined therapy further decreased serum IgE and IL-4 levels when compared to olopatadine or JNJ7777120 monotherapy. Interestingly, IFN-γ and IL-12 show a completely opposite response to H1 and H4 receptor antagonists, increased by H4 receptor antagonist JNJ7777120 and decreased by H1 receptor antagonist olopatadine. Since IFN-γ and IL-12 are typical Th1 cytokines, histamine acts oppositely on the regulation of the Th1 cytokine profile through H1 and H4 receptors. It was reported that H1 receptor knockout mice developed reduced allergen-specific skin reaction, and dendritic cells produced reduced amount of IL-12 upon allergic stimulation (Vanbervliet et al. 2011). There are not enough reports to conclude the effect of allergen-specific skin reaction of the H4 receptor knockout on Th1/Th2 balance. However, it was suggested that the effect of permanent knockout H4 receptor was different from the pharmacological blockade with H4 receptor antagonists (Rossbach et al. 2015). Rossbach et al. discuss in their report that the effect of H4 receptor antagonists was not strong enough to silence H4 dependent signaling. The combined treatment with olopatadine and JNJ7777120 reduces scratching counts and serum IgE levels, with potency comparable to prednisolone (Ohsawa and Hirasawa 2012). Olopatadine, together with JNJ7777120, inhibited thymus and activation-regulated cytokine production in bone marrow-derived mast cells and decreased the infiltration of CD4+ cells in the skin (Ohsawa and Hirasawa 2012). This last report also confirmed that the

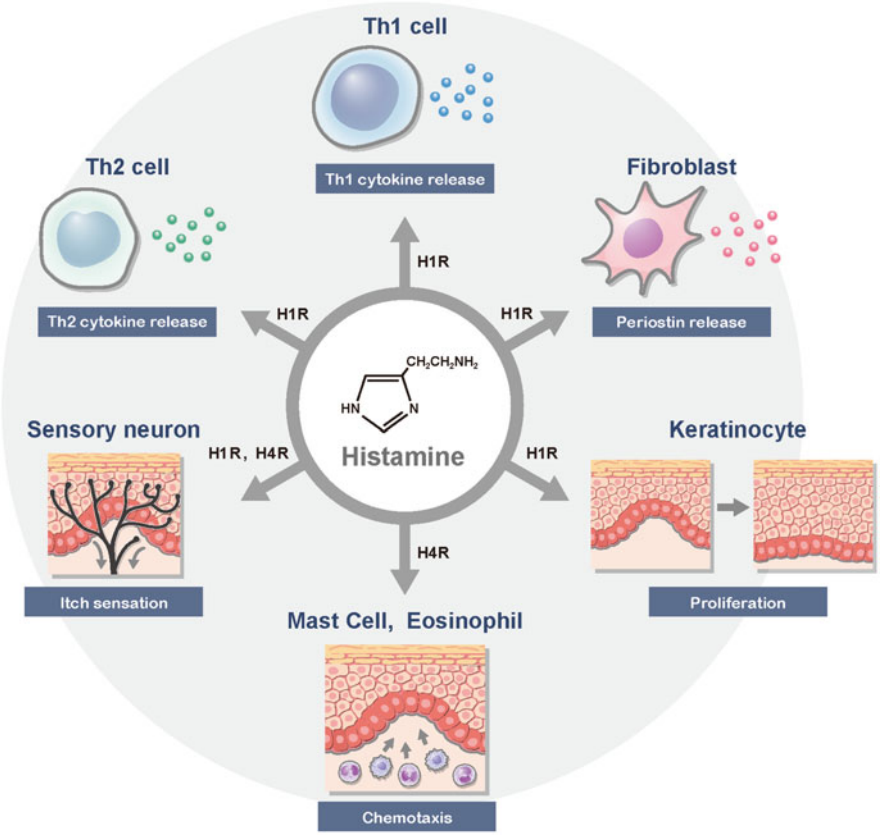

Fig. 1 Increasing effects of histamine through H1 and H4 receptors in skin allergic reaction. Histamine is involved in itch sensation of the sensory neuron via H1 and H4R (Andrew and Craig 2001; Seike et al. 2005b; Rossbach et al. 2011). Keratinocyte is proliferated by histamine via H1R (Seike et al. 2005a; Glatzer et al. 2013). H4R mediates chemotaxis of mast cell and eosinophils (Hofstra et al. 2003; Ling et al. 2004; Seike et al. 2010; Shiraishi et al. 2013). Histamine induces periostin release of fibroblast via H1R (Yang et al. 2014). Th1 (Noubade et al. 2007) and Th2 (Botturi et al. 2010) cytokine releases are induced by histamine via H1R

combined administration of olopatadine and JNJ7777120 inhibited the increase of IL-4 and IL-5 levels in skin lesions. H1 and H4 receptor antagonists synergistically suppressed Th2 cytokine release in the skin in an allergic dermatitis murine model. Combinatory various effects of histamine H1 and H4 receptors were summarized in Fig. 1.

5 Conclusion and Perspective

All of the above studies provide evidence of a pathogenetic and immunomodulatory role of histamine in chronic allergic inflammatory skin diseases. Mainly, H1 and H4 receptors modulate the relevant cell populations by influencing chemotaxis, cytokine release, and itch sensation produced independently or cooperatively. Therefore, a combination of H1 and H4 receptor antagonisms might be a potent therapeutical option for chronic inflammatory skin diseases such as CACD and AD. When new histamine receptor(s) are identified in the future, their therapeutic application and pathophysiological mechanism behind allergic dermatitis should be further investigated for a better clinical application.

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Role of the Histamine H_4-Receptor in Bronchial Asthma

Detlef Neumann

Contents

Abstract

Histamine is a pro-inflammatory mediator with a prominent role in allergic diseases. Antagonists at the histamine receptor subtype 1 are central in anti-allergic therapies, with the exception of allergic asthma, where they are clinically without effect. The latest identified histamine receptor subtype 4, which is expressed mainly in hematopoietic cells, now provides a reasonable target for new therapeutic strategies inhibiting histamine function. The pathophysiology of allergy, esp. allergic asthma, and in its context the effects of antagonists at the histamine receptor subtype 4 in preclinical and clinical settings are discussed in this chapter.

Keywords

Allergy • Asthma • Dendritic cell • Sensitization • T cell

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1 Allergy

Allergy is the inadequate reaction of the immune system to innocuous environmental substances, such as grass pollen, referred to as allergens. Upon contact with the immune system, allergens trigger a complex immune response. In case the immune response is characterized by the excessive production of allergen-specific immunoglobulin E (IgE) and inflammatory symptoms appear immediately after allergen contact, the allergy is classified as a *type 1* hypersensitivity reaction. The pathogenesis of allergy can be subdivided into two phases: initially, the contact between allergen and immune system results in sensitization of the host against the allergen. Within this sensitization phase, allergen-specific T cells, mostly of the Th2 phenotype, and allergen-specific plasma cells that produce allergen-specific antibodies mainly of the IgE isotype are generated. Upon re-exposition of the sensitized host to the allergen, IgE-occupied mast cells and basophil granulocytes (basophils) immediately degranulate, referred to as effector phase. Due to degranulation, a series of preformed mediators such as histamine, heparin, or prostaglandins is released, which eventually elicit the immediate clinical allergic symptoms such as coughing, sneezing, or itching. Also the life-threatening condition of anaphylaxis is mediated by allergic mechanisms, essentially as described.

2 Allergic Response

2.1 Allergic Sensitization

Sensitization occurs upon contact between an allergen and the immune system, mechanistically being highly similar to the process of immunization. If the immune system encounters the allergen for the very first time, the following immune response occurs without any major symptoms for the host. Similar to antigens, allergens are engulfed by mainly dendritic cells (DCs), which together with other cell types constitute the group of professional antigen-presenting cells (APC). Following engulfment, the allergen is processed into fragments, which are loaded onto MHC II molecules. These allergen/MHC II complexes appear on the surface of DC and can be recognized by the T cell receptor (TCR) of allergen-specific naïve $CD4^+$ T helper (Th0) cells (Fig. 1). The interaction between DC and Th0 cells, which also involves cellular contacts by costimulatory molecules, and the predominant presence of interleukin (IL)-4 activate the Th0 cells for polarization into type 2 effector T helper (Th2) cells. Under these conditions, Th1 and Th17 cells, which are able to inhibit Th2 cell activity by the release of specific mediators, are less frequently polarized. Th2 cells are defined by their production of IL-4, IL-5, and IL-13, which besides other functions direct differentiation of allergen-specific B cells into IgE-producing plasma cells (Vroman et al. 2015). Allergen-specific IgE antibodies circulate throughout the body and eventually bind to Fcε-receptors (FcεR) on tissue-resident mast cells and basophils, which are predominantly located

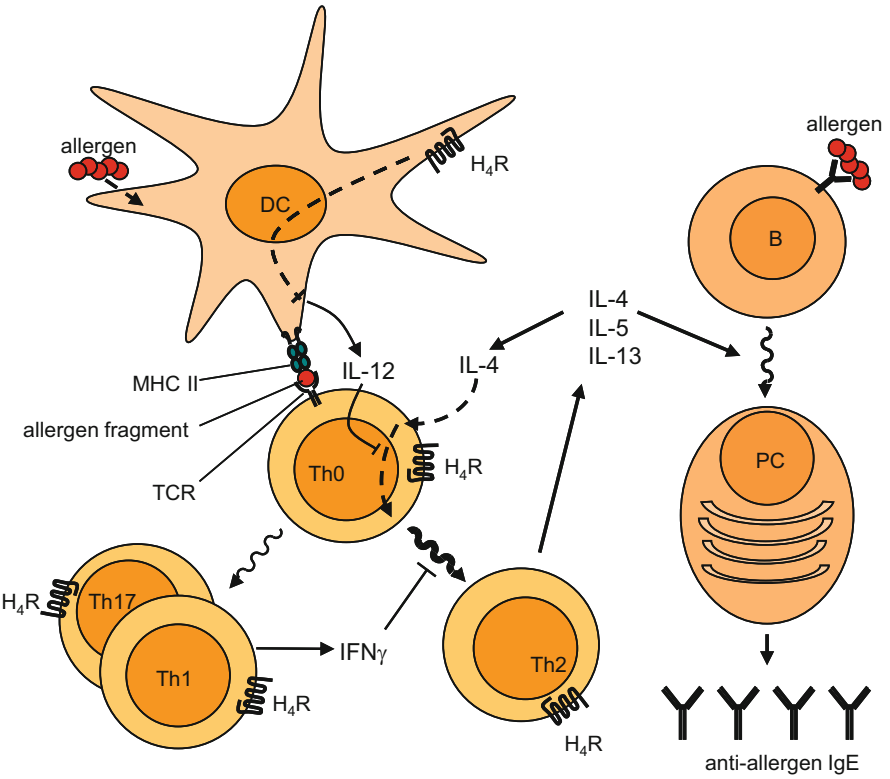

Fig. 1 Allergic sensitization. In the periphery, allergen is sampled by dendritic cells (DC) and then processed into fragments. Allergen-loaded DC migrate to draining lymph nodes, where they present allergen fragments via major histocompatibility complex type II (MHC II) molecules. The allergen fragment/MHC II complex is recognized by specific T cell receptors (TCR) and, thus, activates naïve $CD4^+$ T cells (Th0). Due to a Th2-favoring cytokine milieu, i.e., high concentration of interleukin (IL)-4, Th0 cells mainly polarize into type 2 helper T cells (Th2), which produce the cytokines IL-4, IL-5, and IL-13. Histamine modulates this process via the DC-expressed H_4R, while at least in mice H_4R on T cells do not seem to be involved. Mechanistically it can be hypothesized that DC-expressed H_4R inhibit IL-12 production, which blocks Th2 cell development and favors Th1 cell polarization. Eventually, Th2 cell-derived IL-4 may auto-amplify the polarization of Th2 cells and promotes the development of activated allergen-specific B cells (B) into plasma cells (PC) secreting allergen-specific immunoglobulin (Ig)E

near to the body's surfaces, e.g., bronchial and intestinal mucosae. By this mechanism the mast cell acquires an armed, thus sensitized, status (Reber et al. 2015).

2.2 Allergic Reaction

Upon every other contact to the same allergen, the allergen enters the body's surface and directly binds to the IgE molecules on mast cells and basophils. The

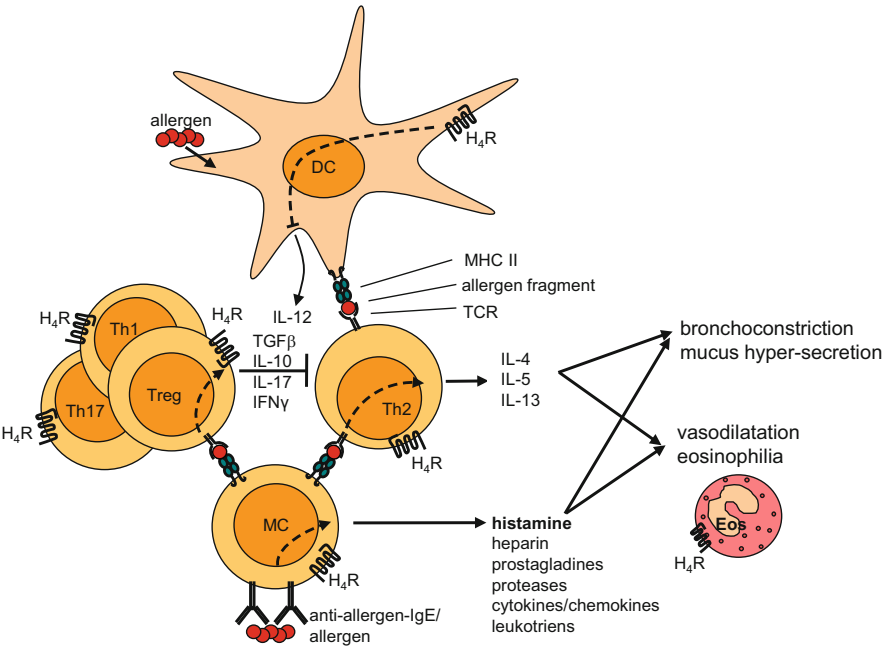

Fig. 2 Allergic reaction (effector phase). Allergen-specific IgE molecules bound to mast cells (MC) which are located near the body's surface bind the allergen and thereby activate the MC to release mediators inducing the allergic symptoms. Histamine via the H_4R regulates migration of MC and thereby may affect this process. In parallel, IgE-bound allergen can be ingested, fragmented, presented via major histocompatibility complex type II (MHC II) molecules, and recognized by T cell receptors (TCR) of effector Type 2 T helper cells (Th2). Similarly, the allergen can be sampled, processed, and presented by local dendritic cells (DC). The activated effector Th2 cell then locally produces large amounts of interleukin (IL)-4, IL-5, and IL-13, which promote the induction of allergic symptoms. Histamine via H_4R may modulate this process since it affects migration of eosinophils, thus the symptom eosinophilia

interaction of multiple IgE molecules with the same allergen leads to cross-linking of FcεR followed by degranulation of the cells (Fig. 2). As a consequence, granule-derived pro-inflammatory mediators such as histamine, proteases, and prostaglandins are released immediately. Moreover, mast cells and basophils start to produce other mediators such as chemokines, cytokines, and leukotriens. These mediators cause allergic symptoms such as redness, rashes, sneezing, and anaphylaxis (Reber et al. 2015). In parallel, DCs sample allergen, process, and present it to effector Th2 cells, a mechanism which can be effectuated also by mast cells after ingestion of IgE-bound allergen. This leads to the local activation of Th2 cells, resulting in the secretion of Th2-type cytokines, supporting the allergic symptoms.

2.3 Allergic Asthma

Asthma is a chronic disease of the airways showing up with very heterogeneous phenotypes, one of which is allergy based (Wenzel 2012; Woodruff 2013). Common features are airway inflammation; airflow obstruction due to airway smooth muscle constriction, mucus hypersecretion, and mucus cell metaplasia; airway hyperresponsiveness (AHR); and airway remodeling. The maturation, activation, and recruitment of eosinophils into the bronchial wall and the airway lumen are key events in the pathogenesis of allergic asthma (Jacobsen et al. 2007; Jacobsen et al. 2014). Eosinophilia in asthma is orchestrated by Th2 cells, mainly through the regulated production of IL-5 (Fig. 2). Moreover, also the innate arm of the immune system, i.e., epithelial cells and group 2 innate lymphoid cells (ILC2), which produce IL-5 and other Th2-type cytokines, too, contribute to eosinophilia in the airways (Liu et al. 2015; Klein Wolterink et al. 2012). If the cellular infiltration in the airways is composed of mostly eosinophils, this is indicative for mild or moderate asthma. In contrast, severe asthma is accompanied by infiltration with eosinophils and also neutrophils. Neutrophils are not activated by Th2-type mechanisms, but mainly by Th17 cells (Cosmi et al. 2011), indicating that in chronic asthma, the sensitization-generated Th2 cell bias is not stable and may shift toward Th1/Th17 cell activation.

3 The Histamine H_4-Receptor

3.1 Identification of the Histamine H_4-Receptor

About 15 years ago, several groups simultaneously discovered the H_4R due to its genomic similarity to the H_3R (Oda et al. 2000; Morse et al. 2001; Liu et al. 2001; Nguyen et al. 2001; Nakamura et al. 2000; Hough 2001). Initially, it was proposed that H_4R are expressed exclusively on cells of hematopoietic origin such as dendritic cells (DCs), T cells, mast cells (MC), and eosinophils (Gutzmer et al. 2002; Hartwig et al. 2015; Hofstra et al. 2003; Reher et al. 2012). This proposal, however, is nowadays being challenged, since evidence is accumulated that H_4R is expressed also by certain cells of non-hematopoietic origin (Adderley et al. 2015; Rossbach et al. 2011; Yamaura et al. 2009). Due to its mainly hematopoietic expression and taking into account that histamine is a pro-inflammatory mediator, it was tempting to speculate that H_4R are involved in inflammation and immune responses. This hypothesis was underscored by the observation that some inflammatory disorders in which histamine is most probably involved cannot be controlled by antagonists selective for H_1R or H_2R. Thus, an effort was made to analyze whether the H_4R is the missing link between histamine and these disorders (Jutel et al. 2002; Venable et al. 2005; Zhang et al. 2007; Seifert et al. 2013). One of these diseases is allergic asthma, where fairly high concentrations of histamine can be detected in the lungs of patients or of model

animals, while the anti-allergic H_1R-selective antihistamines lack any efficacy (Calhoun et al. 1998; Hannon et al. 2001; Sirois et al. 2000).

3.2 Histamine H_4-Receptor Antagonists

The advent of the H_4R solved some issues about unexplainable pharmacological observations made with ligands which have been supposed to be specific for histamine receptor subtypes other than the H_4R. These ligands such as clobenproprit, Rα-methylhistamine, thioperamide, imetit, and 4-methylhistamine were found to possess affinity for and activity on the H_4R as well. R-α-methylhistamine and imetit, both originally described as agonists at the H_3R, are agonists at the H_4R, too, albeit a lesser potency. The same holds true for thioperamide but being an antagonist at both receptor subtypes. The situation of clobenpropit is a bit more complex, since it is antagonistic at the H_3R while it is an agonist at the H_4R. Lastly, the formerly identified H_2R-selective agonist 4-methylhistamine is a bispecific agonist at the H_2R and the H_4R (Seifert et al. 2013).

The first commonly available selective antagonist at H_4R is the compound JNJ 7777120 (5-chloro-2-[(4-methylpiperazin-1-yl)carbonyl]-1*H*-indole) (Thurmond et al. 2004; Seifert et al. 2011; Jablonowski et al. 2003). JNJ 7777120, as well as its analog JNJ 10191584, has become a very useful tool to analyze the H_4R function in vitro and in vivo. In the mouse model of acute peritonitis, JNJ 7777120 prevented the massive neutrophil influx, indicating that in vivo the H_4R function probably is pro-inflammatory (Thurmond et al. 2004). Concerning the use of JNJ 7777120 in vivo, esp. in chronic models, caution has to be paid since JNJ 7777120 possesses an only short half-life time (1–2 h), limiting its effects to only a couple of hours (Neumann et al. 2013). This problem, however, can be solved either by repeated application or by a continuous application of JNJ 7777120 over a prolonged period, e.g., via implanted osmotic pumps. Moreover, based on in vitro data, concerns about its antagonistic, thus anti-inflammatory, function have been raised (Schnell et al. 2011; Rosethorne and Charlton 2011). However, data probably reflecting these concerns in vivo have been documented only once: in the model of experimental autoimmune encephalomyelitis (EAE), where JNJ 7777120 exacerbates the disease (Ballerini et al. 2013), while in all other model systems tested, JNJ 7777120 provided a clear anti-inflammatory effect. The data provided by Ballerini et al. (2013), however, could also point to an anti-inflammatory role of H_4R specifically in EAE and maybe other Th1/Th17-mediated diseases. This interpretation fits well also in the hypothesis that H_4R enhances a Th2-type immune response; thus its inhibition shifts the bias toward Th1 and/or Th17 activity. Finally, although the concerns discussed above have arisen and other ligands selective for the H_4R such as UR-PI376, UR 60427, and ST 1006 have been developed, nowadays JNJ 7777120 has become the gold standard for experimental H_4R antagonism.

As for its use in humans, another complication appears with JNJ 7777120, since it demonstrated signs of adrenal toxicity (as discussed by Rob Thurmond in Chap.

18 of this issue). Thus, a new antagonist, JNJ 39758979, (R)-4-(3-amino-pyrrolidin-1-yl)-6-isopropyl-pyrimidin-2-ylamine, has been developed, which was submitted to clinical trials. JNJ 39758979 showed efficacy in reducing both histamine-induced pruritus in healthy volunteers (Kollmeier et al. 2014) and itch sensitization in atopic dermatitis patients (Ohsawa and Hirasawa 2014). Unfortunately, the phase II trial was terminated due to severe unwanted effects in some of the study patients. Other clinical studies using different H_4R-selective antagonists in a variety of diseases can be found in the database clinicaltrails.gov. These comprise the use of PF-03893787 in allergic asthma, of UR-63325 in allergic rhinitis, and of JNJ 39758979 in persistent asthma. However, although these studies were already completed in the years 2010, 2011, and 2014, respectively, the results obtained are still elusive. A newer study using JNJ 38518168, generically named toreforant, in plaque-type psoriasis is currently (October 2015) in the phase of recruitment. Thus, antagonizing H_4R activity in humans provides a therapeutic benefit, at least against pruritus; however, the advent of an antagonist with the potential to be approved seems to be far away.

This disappointing conclusion may also reflect an additional problem with H_4R ligands and their translation from preclinical, thus, animal models, esp. in mice, to clinical trials. When comparing mouse and human H_4R pharmacologically, it appears that their affinities to selective ligands differ quite massively (Strasser et al. 2013). The human H_4R binds the endogenous ligand histamine with an about 10-times higher affinity ($K_D \sim 7$ nM) than does the murine H_4R ($K_D \sim 60$ nM), while JNJ 7777120 has a comparable affinity to both receptor orthologs ($K_I \sim 5$ nM and 4 nM, respectively). Thus, to compete with histamine at the H_4R, in humans about 10-times higher effective concentrations of JNJ 7777120 would have to be achieved as those determined in mice. Since such differences apply also to other H_4R ligands as well as to other cross-reacting receptor systems, esp. in the histamine-histamine receptor system based on mouse data, it is quite impossible to predict the sum of effects in humans.

3.3 Histamine H_4-Receptor Deficient Mice

Soon after the identification of the H_4R, C57Bl/6 mice lacking expression of this receptor ($H_4R^{-/-}$) were generated by replacing most of exon 1 and part of intron 1 of the Hrh4 gene by a neomycin resistance gene cassette (Hofstra et al. 2003). In mast cells obtained from $H_4R^{-/-}$mice, histamine-induced calcium mobilization and chemotaxis, which both occurred in wild-type mast cells, were absent. Notably, in mouse mast cells degranulation was unaffected by the absence of H_4R expression (Hofstra et al. 2003), while human mast cells seem to directly respond to H_4R activation by degranulation (Jemima et al. 2014). For analyses of allergic diseases, the disrupted Hrh4 locus was backcrossed onto the BALB/c strain (Hartwig et al. 2015; Dunford et al. 2006). Untreated, these mice macroscopically do not demonstrate phenotypical alterations (e.g., viability, Mendelian ratio, gender ratio, growth) compared to their wild-type counterparts (C. Kloth & D. Neumann,

unpublished observations). When applying $H_4R^{-/-}$mice to models of inflammatory diseases, symptoms were generally ameliorated in comparison to wild-type mice (Neumann et al. 2014). However, cellular and molecular mechanisms underlying the protective effect are not well elaborated and may differ depending on the model used.

4 Histamine H_4-Receptor and Asthma

In the lungs of asthma patients as well as of asthma model animals, histamine is detected in fairly high concentrations, indicating its possible pathogenic role. However, the clinically approved antihistamines which block H_1R or H_2R functions lack any effect in asthma. The identification of the pro-inflammatory H_4R thus led to the hypothesis that this receptor subtype is the one mediating the histamine effect in asthma. Genetic evidence for a role of the H_4R in human asthma has been provided by Simon et al. (2012), who demonstrated that some polymorphisms within the Hrh4 gene are associated with asthma induced by infection (Simon et al. 2012). As already discussed above, clinical trials treating asthma patients with antagonistic H_4R ligands have been performed; however, results of these studies have not been published so far (Salcedo et al. 2013). Thus, concerning human asthma, the functional involvement of the H_4R and its possible role as drug target is still elusive.

In mice ovalbumin (OVA)-induced asthma is a commonly used model presenting some features closely resembling human allergic asthma, i.e., eosinophilic inflammation and AHR. Using this model, the contribution of H_4R to the pathophysiology was intensively studied (Hartwig et al. 2015; Neumann et al. 2013; Dunford et al. 2006; Beermann et al. 2012; Cowden et al. 2010). When H_4R function in mice was blocked, either by treatment with a selective antagonist or by genetic ablation, asthmatic symptoms were significantly reduced. Thus, as expected from other models of inflammatory diseases, in the acute asthma model, H_4R demonstrates a pro-inflammatory function, too. Animal models of allergic asthma bear the advantage that the two phases sensitization and allergic reaction, the latter one also being referred to as effector phase, can be clearly distinguished. Thus, in contrast to asthma patients, in animal models also, the sensitization phase can be followed and relevant mechanisms can be analyzed (Fig. 1). Naturally, mice do not develop asthma. Thus, an asthma-like disease has to be experimentally induced, i.e., for sensitization mice are injected with the experimental allergen OVA, which may be formulated with an adjuvant such as aluminum hydroxide (alum), in order to initiate an immune reaction against OVA (Fig. 3). The resulting T cell-dependent immune reaction can be boosted after approx. 2 weeks by an additional systemic injection of OVA. In order to trigger the effector phase, the sensitized mice are challenged topically in the lung either by inhalation of nebulized OVA or by intranasal delivery of an OVA-containing solution (Fig. 3). Notably, in BALB/c mice this treatment regime using alum/OVA for sensitization

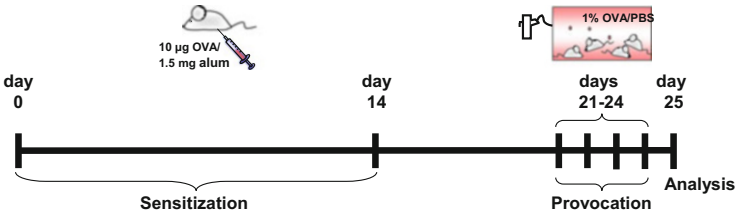

Fig. 3 Ovalbumin-induced acute asthma in mice. To model human asthma, mice are sensitized to ovalbumin (OVA) by two intraperitoneal injections of OVA formulated with aluminum hydroxide (alum) on days 0 and 14. One week later, on days 21–24 mice are daily exposed to a nebulized phosphate-buffered saline (PBS) solution containing OVA. Finally, the mice are analyzed on day 25

most probably results in an asthma-like disease which is independent of mast cells (Lei et al. 2013).

Using JNJ 7777120 or JNJ 10191584, the functional relevance of H_4R in both sensitization and effector phase of an acute asthmatic reaction in mice was demonstrated (Beermann et al. 2012; Cowden et al. 2010; Dunford et al. 2006; Thurmond et al. 2008). Application of the H_4R antagonists exclusively during either the sensitization or the effector phase both ameliorated the symptoms of acute asthma and the signs of Th2-type inflammation, indicating that histamine and H_4R are involved in both phases of the asthma model. Addition of an H_1R antagonist to the H_4R antagonist modified the effect obtained with the H_4R antagonist alone, however, dependent on the timing of application. When applied during sensitization, the H_1R antagonist enhanced the effect of the H_4R antagonist, while in the effector phase it reduced it (Beermann et al. 2012). Thus, the murine H_1R and H_4R functions can interact with each other in at least two different qualities, indicating that more than one possible mechanism exists for such interaction.

Cells typically involved in allergic asthma are mast cells and eosinophils. Since both cell types are responsive to H_4R stimulation (Hofstra et al. 2003; Reher et al. 2012; Jemima et al. 2014; Desai and Thurmond 2011), this may provide an explanation why H_4R antagonists are effective in reducing the asthmatic phenotype in mice. Interestingly, the JNJ 7777120 effect on OVA-induced asthma is still observed in mast cell-deficient WBB6F1-Kit^W/$Kit^{W\text{-}v}$ mice (Dunford et al. 2006). This was to be expected, since, in contrast to humans, the lungs of mice, esp. parenchyma and alveoli, are sparsely populated with mast cells. Due to these anatomical bases, the contribution of mast cells to asthmatic responses in mice strongly depends on the protocol used for induction, and, as discussed above, the protocol applied by Dunford et al. (2006) induces a rather mast cell-independent airway inflammation (Lei et al. 2013). Thus, a mast cell-mediated H_4R effect in mouse experimental asthma cannot definitively be excluded; it may be just absent in the model applied.

For human eosinophils, H_4R stimulation has been shown to be a chemotactic signal (Reher et al. 2012; Thurmond et al. 2014; Ling et al. 2004). Although very

likely, the proof whether this observation holds true also for mouse eosinophils is still lacking.

The OVA-induced asthma model is dependent on T cells (Coyle et al. 1995). Direct effects of the H_4R on T cell cytokine production cannot be excluded, at least in humans (Jutel et al. 2001, 2002, 2005). However, in mouse CD4 T cells, H_4R activation does not affect IFNγ production upon in vitro stimulation (Vauth et al. 2012), while it is rather H_2R, which mediates such effects of histamine (Vauth et al. 2012; Krouwels et al. 1998). Accordingly, lack of H_4R expression on CD4 T cells in sensitization of a combined in vitro/in vivo mouse asthma model seems to be without any effect on the disease (Hartwig et al. 2015).

In vitro data point to a potential role of H_4R on T cell priming by DC (Dunford et al. 2006), and in an in vitro/in vivo asthma model, it was demonstrated that H_4R on DC accounts for the ameliorating effect of H_4R blockade (Hartwig et al. 2015). Thus, the effect of H_4R on CD4 T cell polarization toward the Th2 phenotype is rather indirectly mediated via DC, at least in the mice asthma model (Fig. 1). In human DC, a role for H_4R was evaluated in vitro and indicates that it regulates mediator expression as well as migratory behavior (Gschwandtner et al. 2010, 2011). Strikingly, in human DC, H_4R activation inhibits the expression of IL-12 (Gutzmer et al. 2005). Thus, by inhibition of H_4R activation, the Th1-inducing cytokine IL-12 is produced in higher amounts, probably shifting the T cell polarization bias from the Th2 toward the Th1 phenotype, eventually reducing an allergic phenotype (Figs. 1 and 2). Whether this indeed holds true also in vivo, thus in human asthma patients still has to be elaborated.

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