Signal transduction of the histamine H3 receptor

Bongers, G.M.

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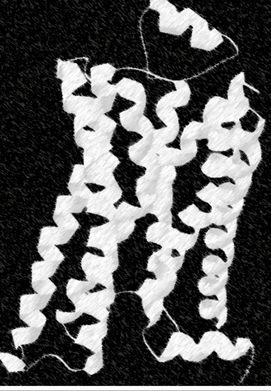

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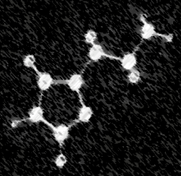

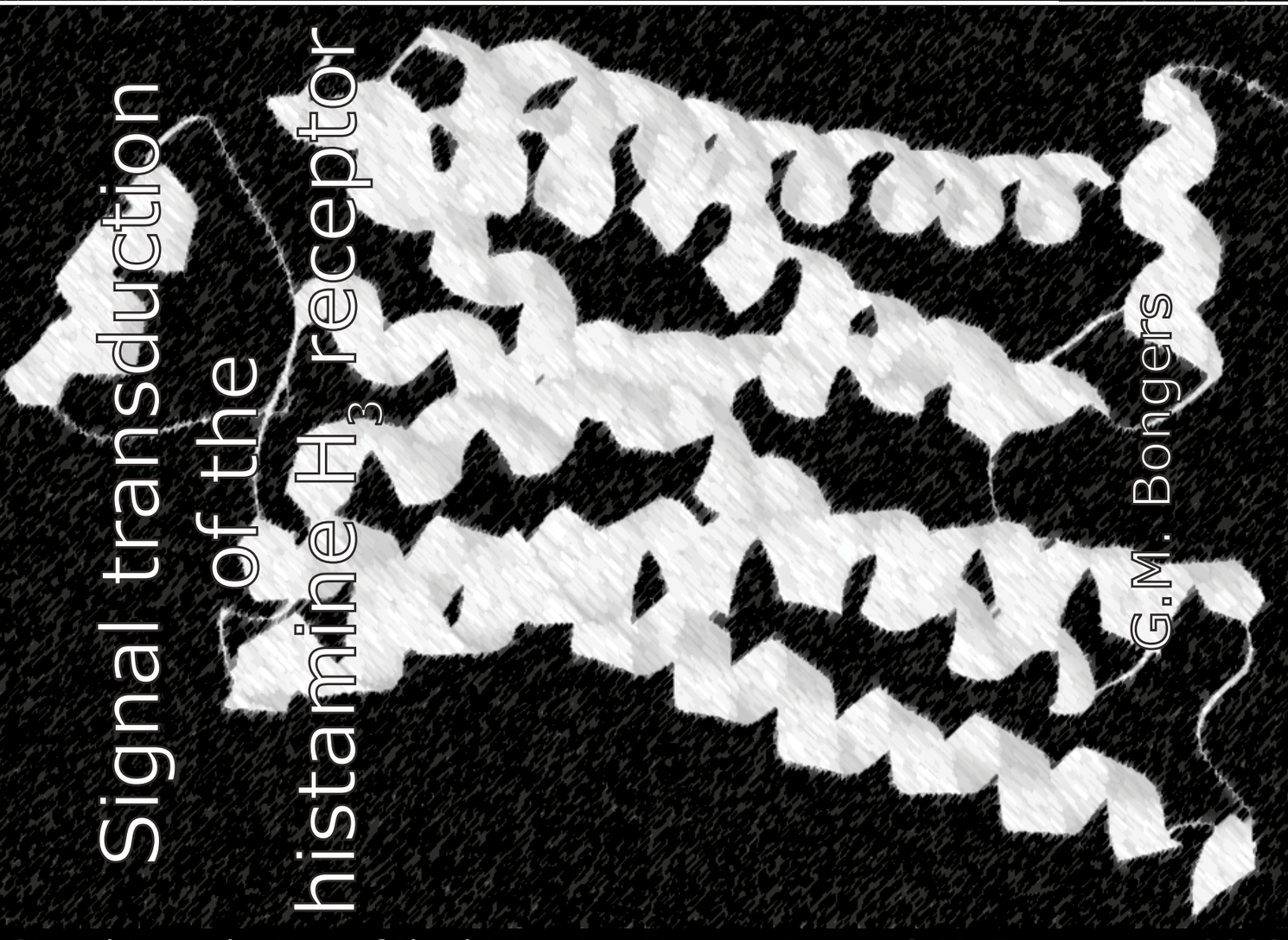

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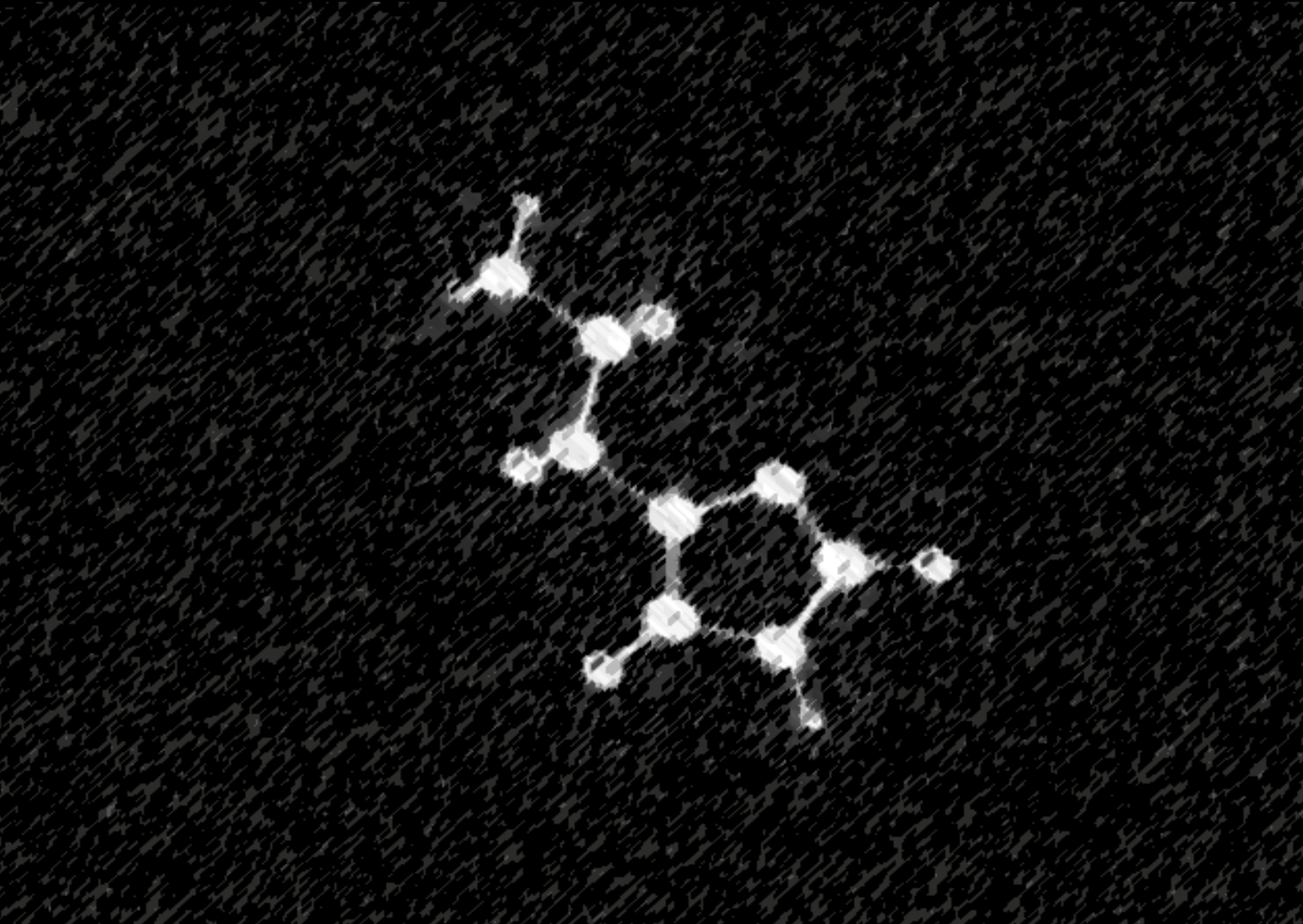

Signal transduction of the histamine H_3 receptor

Gerold Bongers

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Signal transduction of the histamine H_3 receptor

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door

Gerardus Martinus Bongers

geboren te Oss

promotor: prof.dr. R. Leurs
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Chapter 1

Introduction

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are a large family of membrane-bound proteins that convert diverse stimuli like odors, photons, neurotransmitters, hormones, peptides and proteases, via guanine nucleotide-binding proteins (G-proteins) into intracellular responses. GPCRs are characterized by seven alpha helical transmembrane (TM) domains and are found in eukaryotes, including yeast, plants, choanoflagellates, and animals. They are involved in numerous physiological processes like smell, taste, vision, behavior and mood, regulation of the immune system and autonomic nervous system transmission. GPCRs are considered as an attractive drug target by the pharmaceutical industry, because GPCRs are involved in the regulation of almost every major mammalian physiological process and are readily accessible to drugs due to their localization on the cell surface. In fact, 30% of all drugs on the market are targeting GPCRs, among these are several block-buster drugs like clopidogrel (Plavix®), Fexofenadine hydrochloride (Allegra®), quetiapine (Seroquel®) and metoprolol (Lopressor® or Seloken® (Wise et al., 2002; Service, 2004).

Structural features of GPCRs

Based on phylogenetic analysis according to the GRAFS classification system GPCRs can be divided into five main classes: the glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin (Figure 1) (Fredriksson et al., 2003).

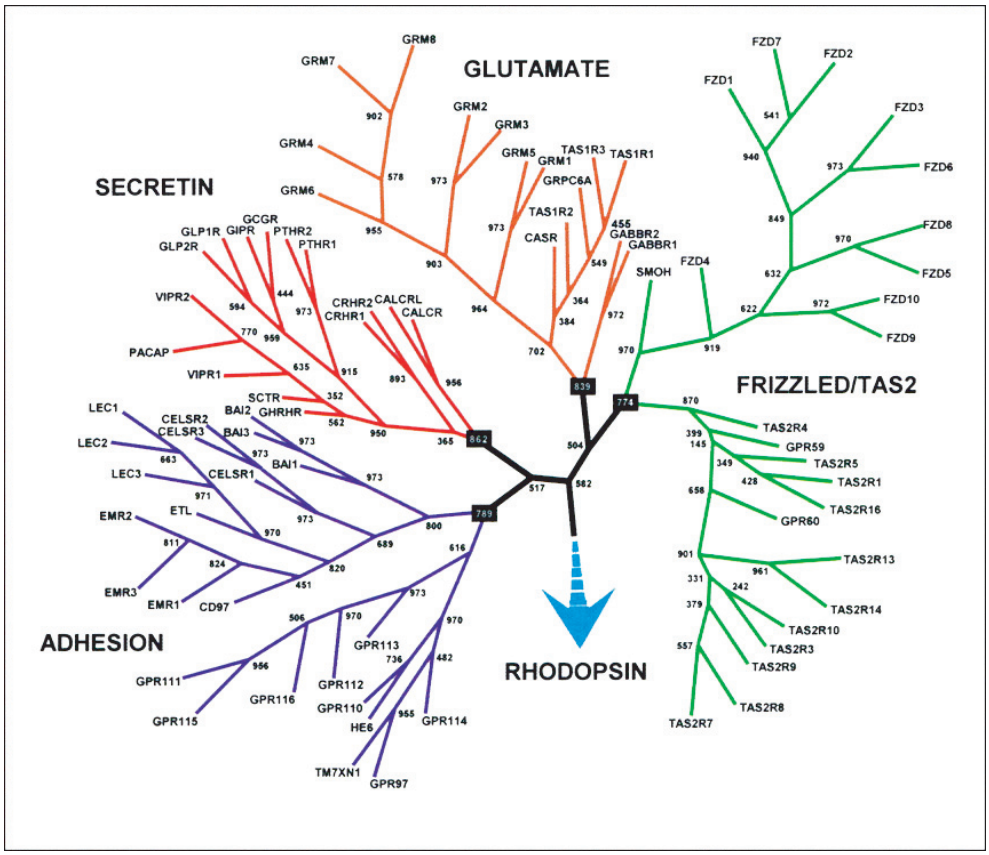

Figure 1. Phylogenetic relationships between the GPCRs in the human genome, for the rhodopsin family see Figure 2. Adapted from (Fredriksson et al., 2003).

The rhodopsin-class of GPCRs is the largest class and can be further divided into four main groups, α, β, γ and δ with 13 sub-branches (Figure 2). The rhodopsin class represents receptors like the olfactory receptors (δ-group), chemokine receptors (γ-group), peptide receptors (β-group) and the biogenic amine receptors (α-group), and is characterized by some highly conserved motifs like the (D/E)R(Y/F) motif at the end of TM3 and the NSxxNPxxY motif in TM7.

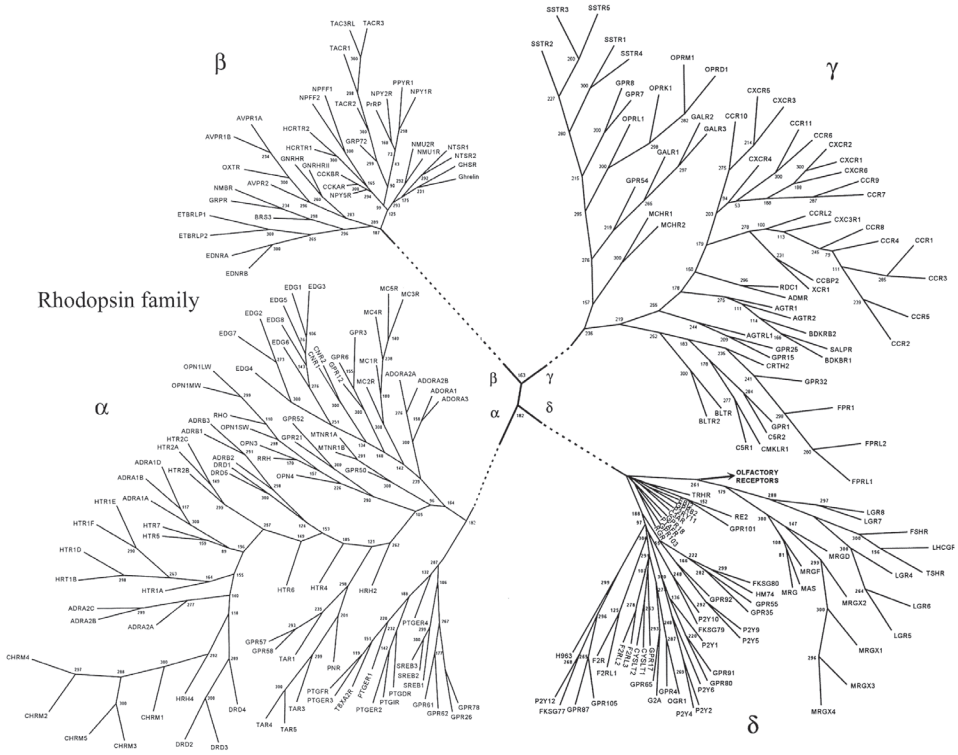

Figure 2. The phylogenetic relationships between GPCRs in the human rhodopsin family. The rhodopsin family of GPCR can be subdived into four main groups namely: α, β, γ and δ with 13 sub-branches. Adapted from (Fredriksson et al., 2003).

Structural models of GPCRs are so far mainly based on the crystal structure of bovine rhodopsin (Palczewski et al., 2000). More recently crystal structures became available of the β_1-adrenergic receptor and β_2-adrenergic receptor (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007; Warne et al., 2008). These crystal structures show that GPCRs consist of a single strand of amino acids with an extracellular N-terminal tail, seven alpha helical domains that traverse the cellular membrane and an intracellular C-terminal tail (Figure 3). Due to its structural features, GPCRs are also know as seven transmembrane receptors, 7TM receptors or heptahelical receptors.

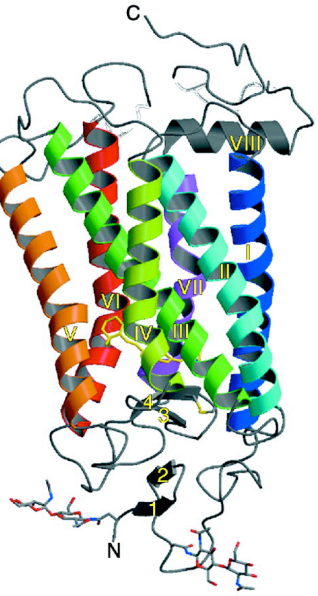

Figure 3. Crystal structure of rhodopsin showing the extracellular loops and the N-terminal tail (bottom), seven alpha helical transmembrane domains (middle), intracellular loops and alpha helical C-terminal tail (top). Adapted from (Palczewski et al., 2000).

G-protein dependent signaling

The heterotrimeric G-protein consists of a Gα subunit and a tight complex formed from a Gβ-subunit and a Gγ-subunit. At least 17 Gα, 5 Gβ and 14 Gγ-subunits are know (Wettschureck and Offermanns, 2005), leading to a huge diversity of 1190 theoretical combinations, although not all combinations might be found *in vivo*.
In the basal state the guanine diphosphate (GDP)-bound Gα-protein is associated to the βγ-complex. Coupling of the heterotrimeric protein to an active receptor promotes the exchange of GDP with guanine triphosphate (GTP) on the Gα-subunit and subsequently leading to dissociation of the Gα-subunit and βγ-complex from the receptor as well as dissociation of the Gα-subunit from the βγ-complex. The free Gα-subunit and βγ-complex are now able to modulate a variety of effectors. Termination of the signal is induced by the hydrolysis of GTP by the inherent GTPase activity of the Gα-subunit and the subsequent re-association of the GDP-bound Gα-subunit with the βγ-complex, which can enter a new activation cycle when bound to an activated receptor (Wettschureck and Offermanns, 2005). However, more recently this scheme was shown to be more complex as it has become apparent that dissociation of G-proteins into their subunits is not always essential for their mechanism of action (Klein et al., 2000; Levitzki and Klein, 2002;

Bunemann et al., 2003; Frank et al., 2005). Additionally, various effectors, among which the so-called regulators of G-protein signaling (RGS), accelerate the deactivation of the G-protein mediated signaling by enhancing the GTPase activity on the Gα-subunit (Abramow-Newerly et al., 2006b).

The Gα-subunits can be divided into four main families, the $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$ consisting of various members with different expression patterns (Milligan and Kostenis, 2006). The $G\alpha_s$-proteins are ubiquitously expressed and couples GPCRs to adenylyl cyclase (AC) resulting in an increase in intracellular cAMP levels. The widely expressed $G\alpha_q$-proteins couples receptors to the β-isoforms of phospholipase C (PLC), which catalyses the conversion of phosphatidylinositol 4,5-biphosphate (PIP_2) into the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which in turn increase the intracellular calcium concentration ($[Ca^{2+}]_i$) or activate protein kinase C (PKC), respectively. GPCRs that couple to $G\alpha_q$-proteins often activate the ubiquitously expressed $G\alpha_{12/13}$-proteins as well. The characterization of $G\alpha_{12/13}$-mediated pathways is hampered by the lack of specific inhibitors and promiscuous nature of the GPCRs that couple to these Gα-proteins, however, studies have shown $G\alpha_{12/13}$-proteins activate various signaling effectors including phospholipase A_2 (PLA_2), Na^+/H^+-exchanger (NHE) and Rho-guanine nucleotide exchange factor (RhoGEF)-mediated activation of RhoA (Kurose, 2003; Riobo and Manning, 2005). The pertussis toxin (PTX) sensitive $G\alpha_{i/o}$-proteins are widely expressed and have been shown to inhibit various types of AC and thereby lower the intracellular cAMP levels. Due to the relative high expression of $G\alpha_{i/o}$-proteins, activation leads to a high release of βγ-subunits and these βγ-subunits are able to activate various signaling cascades. In fact, the predominantly neuronally-expressed $G\alpha_o$-proteins might induce their effect mainly through the release of βγ-subunits (Wettschureck and Offermanns, 2005). The βγ-subunit mediated signaling processes include; inhibition of AC type I and voltage-dependent Ca^{2+} channels (VDCC) and activation of AC type II/IV/VII, PLC-β, G protein-regulated inward rectifier potassium channels (GIRK) and G-protein regulated kinases (GRK) (Clapham and Neer, 1997).

G-protein independent signaling

More recently it has become apparent that besides G-protein dependent signaling, GPCRs are able to modulate various physiological responses in a G-protein independent manner. Hence, 7TM receptors would be a more appropiate name for GPCRs.

One of the most investigated G-protein independent signaling of GPCRs is the arrestin-mediated signal transduction. Initially, arrestins were found to be involved

in the inactivation of GPCR signaling. The arrestin mediated inactivation occurs when activated receptors are phosphorylated by G protein-coupled receptor kinases (GRKs). The subsequent binding of arrestins to the phosphorylated intracellular loop 3 (IL3) and carboxyl-terminal tail (C-tail) promotes receptor recycling via endocytosis and interaction with the clathrin assembly machinery. Besides a role of arrestin in the inactivation of a GPCR, they have also been show to act as an adaptor protein to recruit the tyrosine kinase Src to the clathrin-coated pits and thereby mediates the activation of MAP kinase (Shenoy and Lefkowitz, 2005; Violin and Lefkowitz, 2007). Additionally, GRKs themselves have also been show to act as signaling intermediates and have been shown to phosphorylate key nonreceptor substrates, such as tubulin, and to recruit key signaling intermediates to GPCRs at the plasma membrane, like ARF GTPase-activating protein (ARF GAP) (Premont and Gainetdinov, 2007). Besides arrestins, two proteins were identified as important proteins interacting with the 3IL, namely the scaffolding proteins 14-3-3 and spinophilin (Prezeau et al., 1999; Richman et al., 2001; Brady and Limbird, 2002; Abramow-Newerly et al., 2006b; Wang and Limbird, 2007).

Spinophilin, also know as Neurabin II, is a ubiquitous protein highly enriched in dendritic spines (Allen et al., 1997). It is a multi-domain protein with an actin-binding domain, a protein phosphatase 1 (PP1) binding domain, a regulatory domain, a PSD-95/Discs large/ZO-1 homology (PDZ) domain and three coiled-coil domains. Spinophilin is implicated in synaptic plasticity, spine morphogenesis and neuronal migration through its interactions with PP1 glutamate receptors and F-actin, (Satoh et al., 1998; Yan et al., 1999; Feng et al., 2000; Tsukada et al., 2005; Tsukada et al., 2006). Amino acids 151-444 of spinophilin have been shown to specifically interact with the 3IL of $G\alpha_{i/o}$-coupled GPCRs (MacMillan et al., 1999; Smith et al., 1999; Richman et al., 2001; Wang et al., 2005). Interactions of spinophilin with a GPCR requires the N- and C-terminal ends of the 3IL (Wang and Limbird, 2002), a region that is also involved in the binding of GRK2 (Pao and Benovic, 2005). Furthermore, spinophilin has been shown regulate GPCR signaling duration and response sensitivity by competing with arrestins for binding to the 3IL (Figure 4) (Wang et al., 2004). Spinophilin also recruits RGS proteins to GPCRs and thereby was shown to markedly increase the inhibition of Ca^{2+} signaling by the α-adrenergic receptor. Notably, a constitutively active mutant of α-adrenergic receptor did not bind spinophilin and was relatively resistant to inhibition by RGS (Wang et al., 2005; Wang et al., 2007).

The seven known mammalian isoforms of the 14-3-3 proteins (β, γ, ε, σ, τ and ζ) are ubiquitously expressed and function primarily as chaperones, adaptors and scaffolds (Jones et al., 1995; Dougherty and Morrison, 2004; Wilker and Yaffe, 2004). The 14-3-3 proteins have been shown to bind diverse (~200) signaling

molecules including protein kinases, members of the ras signaling pathway, RGS proteins, cell cycle component and GPCRs (Aitken, 1996; Fu et al., 2000). Initially it was thought that a phosphorylated serine/threonine motif (RSXpSXP or RXY/FXpSSXP) was necessary for recognition, but variations on this motif, including phosphorylation-independent binding, have been observed (Muslin et al., 1996; Aitken et al., 2002; Pozuelo Rubio et al., 2004). The effects of 14-3-3 on its binding partners are thought to either lead to a conformational change, or to a physical occlusion of sequence-specific or structural protein features or to a scaffolding (Bridges and Moorhead, 2005). Interaction of 14-3-3 with GPCRs occurs within the 3IL (Prezeau et al., 1999) and it has been shown to compete with spinophilin and arrestins for binding to the 3IL (Wang and Limbird, 2002). Additionally, 14-3-3 proteins have been shown to decrease the inhibitory effects of RGS proteins and thereby affecting GPCR signaling (Benzing et al., 2000; Benzing et al., 2002; Niu et al., 2002; Abramow-Newerly et al., 2006a; Abramow-Newerly et al., 2006b). Whether interaction of 14-3-3 proteins to either a GPCR or RGS is interrelated remains to be determined (Prezeau et al., 1999; Couve et al., 2001; Brady and Limbird, 2002).

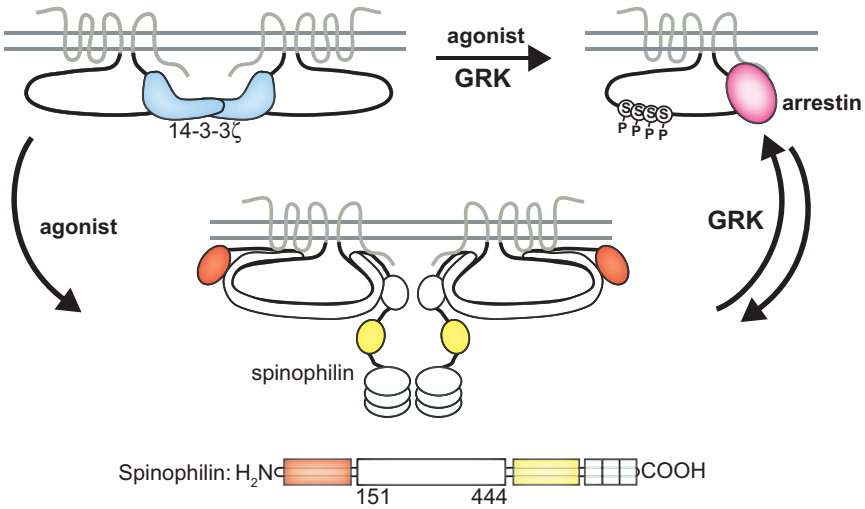

Figure 4. Proposed regulatory cycle for interactions of spinophilin, 14-3-3 and arrestin with the 3IL of a GPCR. Interactions with 14-3-3 likely occur when the receptor is unoccupied and might contribute to receptor targeting and retention at the cell surface. The functional role of 14-3-3 interactions has not been established. Agonist occupancy can stabilize interactions with arrestin and spinophilin, depending on the phosphorylation state of the receptor. Arrestin preferentially binds to GRK-phosphorylated receptors, whereas spinophilin prefers to bind to the non-phosphorylated receptor. Spinophilin and arrestin compete for interaction with the 3IL. Adapted from (Wang and Limbird, 2007).

Also, the Janus kinase (Jak) family of kinases is recognized as an important G-protein independent signaling pathway that may be activated by GPCRs. These receptor-associated tyrosine kinases were originally found to bind and phosphorylate dimeric cytokine receptors and thereby enhance the recruitment signal transducers and activator of transcription (STAT), which in turn are phosphorylated by Jak and subsequently dissociates from the receptor-complex and translocate to the nucleus. Besides binding to cytokine receptors, coimmunoprecipitation experiments revealed a direct interaction with GPCRs and specific Jak and STATs. Additionally, GPCR stimulation was found to lead to phosphorylation of STAT3 (Williams, 1999).
Furthermore, the EVH1 domain of Homer interacts with a proline-rich motif (PPXXFR, e.g. the homer 'ligand') that is present is the C-terminal tails of some GPCRs. The binding of a homer dimer with GPCR is thought to subsequently form a physical coupling with IP_3R and thereby modulates the $[Ca^{2+}]_i$ levels (Shiraishi-Yamaguchi and Furuichi, 2007).
Additionally, GPCRs are known to modulate the Na^+/H^+ exchanger (NHE) activity and this is suggested to be dependent on the G-protein mediated modulation of protein kinase A (PKA) or extracellular signal-regulated kinases (ERK) (Avkiran and Haworth, 2003). However other studies show that this GPCR-mediated modulation of NHE activity is G-proteins independent and it is suggest that a direct interaction of the Na^+/H^+ exchanger regulatory factor (NHERF) with a GPCR plays an important role receptor-mediated regulation of NHE (Hall et al., 1998; Weinman et al., 2006).

Constitutive activity of GPCRs

The classical paradigm of GPCR activation is that agonist binding induces a conformational changed that enhances G-protein coupling and thereby induces a cellular response. Antagonists inhibit the agonist mediated responses, but have no effect themselves. However, most GPCRs, if not all, show some ligand independent activity (Bond and Ijzerman, 2006). This so called constitutive activity is especially apparent in recombinant systems with often relatively high expression levels of the GPCR. In these recombinant systems most 'antagonists' are actually found to be inverse agonists, because they inhibit the constitutive activity of the GPCR. A neutral antagonist, e.g. a compound that has no effect on its own, is rare and most formely know 'antagonists' have been reclassified as inverse agonists (Kenakin, 2004). Constitutive activity in native systems is more difficult to show due to the effect of endogenous agonists on the basal activity of the GPCR. The histamine H_3 receptor is actually one of the best examples for which constitutive activity has been shown in native tissue. Morisset et al. showed that the protean

agonists proxyfan could inhibit both the activity of the agonist imetit and the inverse agonist thioperamide on the autoreceptor mediated [^{3}H]histamine release and guanosine 5'-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding in tissue of rat brain, but to has no effect on its own (Morisset et al., 2000; Moreno-Delgado et al., 2006a).

Constitutive activity of a GPCR can be enhanced by mutations, single nucleotide polymorphisms (SNPs), splice variant or receptor autoantibodies (Bond and Ijzerman, 2006). Some SNPs in rhodopsin, the thyroid-stimulating hormone receptor (TSHR), luteinizing hormone/chorionic gonadotropin receptor (LHCGR), or the follicle-stimulating hormone receptor (FSHR), induce constitutive activity and are associated with certain pathophysiological conditions (Smit et al., 2007).

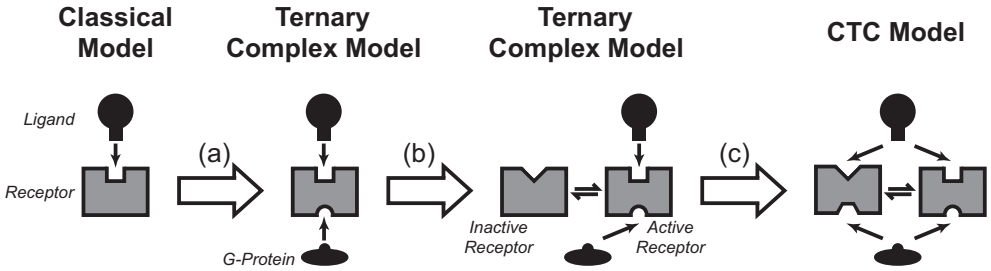

Figure 5. Different mechanistic models. (a) G-protein can couple with receptor. (b) The receptor occurs in two states, the ligand has different affinities for each. G-protein couples only with the receptor in its active conformation. (c) The G-protein can couple with the receptor in either state, but has different affinities for each. Adapted from (Weiss et al., 1996a).

Several mechanistic models are developed to explain receptor-ligand interactions (Figure 5). The simplest of these models is Clarks classical model (Clark, 1937) that consisted of two elements, a ligand (L) and a receptor (R). In this model the ligand is assumed to bind to a single site on the receptor and according to the laws of mass action the ligand and receptor form a receptor-ligand complex. Consequently, two receptor species are present at equilibrium the free receptor (R) and the receptor-ligand complex (LR). To account for certain experimental observation with G-proteins (Rodbell et al., 1971; Sternweis et al., 1981) these models had to be adjusted and the G-protein (G) was introduced as a third component (Kenakin, 1989; Kenakin and Morgan, 1989). In this ternary complex model, the receptor is assumed to have two binding sites, one for the ligand and one for the G-protein, as such the receptor at equilibrium can exists as four different species, R, LR, RG and LRG (Cuatrecasas, 1974; De Lean et al., 1980). The observation that GPCRs can be constitutively active led to the proposal of an extended ternary complex model in which the receptor can be either active (R_a) or

inactive (R_i). In this model receptor activation is a necessary precondition for G-protein coupling and hence at equilibrium six receptor species can be envisioned, R_i, R_a, LR_i, LR_a, R_aG, and LR_aG (Samama et al., 1993). This extended ternary complex model is regarded by some as incomplete and therefore a cubic ternary complex (CTC)-model was proposed to permit G-proteins to interact with receptors in both their active and inactive states. The CTC-model account for all possible combinations in a ternary system and thus has s total of eight receptor species at equilibrium R_i, R_a, LR_i, LR_a, R_aG, LR_aG, R_iG, and LR_iG (Figure 6) (Weiss et al., 1996c; Weiss et al., 1996a; Weiss et al., 1996b). In this model an inverse agonist either uncouples the GPCR from the G-protein or promotes a G-protein coupled non-signaling state (Costa and Cotecchia, 2005).

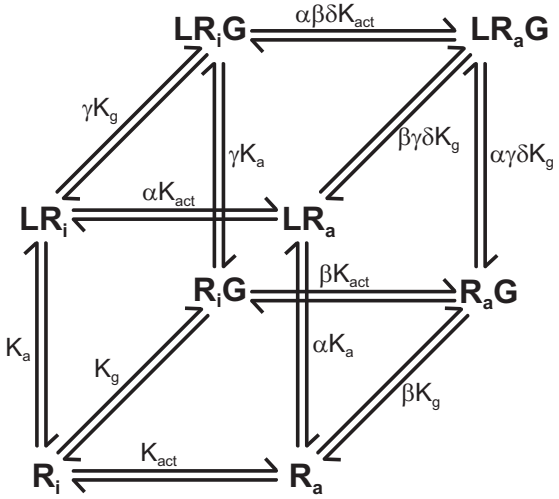

Figure 6. CTC-model with equilibrium association constants in which; K_a and K_g represent the association constants for a ligand (L) and G-protein (G), respectively; K_{act} is the equilibrium constant for the activation of the receptor (R); α and γ represent the effect of ligand binding on activation and G-protein coupling of the receptor, respectively; β represent the effect of receptor activation on G-protein coupling and δ represents the extent to which the joint effect of any two (receptor activation, G-protein coupling or ligand binding) varies the level of the third. Adapted from (Weiss et al., 1996b; Weiss et al., 1996a; Weiss et al., 1996c).

Histamine receptors

The histamine receptors are membrane bound proteins that belong the the superfamily of the GPCRs and more precisly to biogenic amine receptors (α-group)

in the rhodopsin-family of GPCRs. The biogenic amine that endogenously activates these histamine receptors is histamine.

Histamine and the discovery of the histamine receptors

Histamine (2-(3H-imidazol-4-yl)-ethylamine, Figure 7) can be taken up from food as well as being formed through decarboxylation of the amino acid histidine. This reaction is catalyzed the enzyme L-histidine decarboxylase (HDC), an essential determinant of brain histamine levels (Kollonitsch et al., 1978; Green et al., 1987). Synthesis of histamine in the periphery occurs in periphery mast cells, lymphocytes, and gastric enterochromaffin-like cells. In the central nervous system (CNS) synthesis of histamine is restricted to a population of neurons located in the tuberomammillary nucleus of the posterior hypothalamus Formed histamine is subsequently either stored in mast cells, basophiles or neuronal cells. Histamine is metabolized by two main catabolic pathways, the methylation by histamine N-methyltransferase and oxidative deamination by diamine oxidase. The biological effects of histamine in which already observed early on in experiments were injection of the biogenic amine were found to produce the same effect as seen in many allergic reactions (Dale and Laidlaw, 1910a). As early as 1937 the first evidence for a histamine receptor was provided by Bovet and Staub, who discovered the first antihistamine thymoxidiethylamine, that was capable of preventing anaphylactic shock in animals which, when not neutralized, was fatal. (Bovet and Staub, 1937). The discovery by Ash and Schild in 1966 that antihistamines, like mepyramine (Figure 8), could block certain pharmacological actions of histamine on symptoms of allergic reactions, but not the effects on the gastric acid secretion led the hypothesis that there were at least two subtypes of histamine receptors (Ash and Schild, 1966). This was further corroborated by the finding that burimamide (Figure 9) selectively antagonized the histamine mediated effects on the gastric acid secretion (Black et al., 1972b). The histamine mediated auto-inhibition on release of brain release was shown to by mediated by a third class of histamine receptors that could be pharmacologically differentiated from the heretofore known histamine H_1 receptor (H_1R) and histamine H_2 receptor (H_2R) (Arrang et al., 1983). The histamine H_3 receptor (H_3R) was definitely confirmed by the first selective and potent H_3R antagonists thioperamide (Figure 10) (Arrang et al., 1987). The last member of the histamine receptor family was originally cloned as an orphan receptor, but based on its high sequence homology to the H_3R was found to respond to histamine and confirmed to be a fourth histamine receptor, the histamine H_4 receptor (H_4R) (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). These four histamine receptors are all GPCRs.

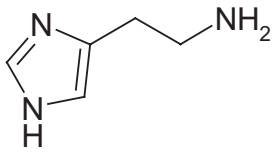

Figure 7. Chemical structure of histamine (2-(3H-imidazol-4-yl)-ethylamine).

Histamine H_1 receptor

The histamine H_1 receptor (H_1R) is found mainly on smooth muscle cells, endothelium and in the CNS. Its physiological role includes vasodilatation, bronchoconstriction, smooth muscle activation, separation of endothelial cells (responsible for hives), pain and itching due to insect stings. Inverse agonists for the H_1R, commonly know as antihistamines, are used for the treatment of allergic rhinitis, skin irritations and asthma (Hill et al., 1997). After the first antihistamine thymoxyethyldiethylamine, ethylenediamines (e.g. mepyramine) were the first clinically developed H_1R antagonists. Like other first generation H_1R antagonists mepyramine suffered from sedation as a side effect. Actually, these compounds are now used in many sleeping-aid preparations. Second generation antagonists for the H_1R, e.g the piperazine cetirizine, have a reduced occurrence of adverse drug reactions due to a decreased brain penetration and increase H_1R selectivity (Kay, 2000).

The bovine H_1R cDNA was cloned from a cDNA library of bovine adrenal medulla and was the first H_1R gene to be cloned (Yamashita et al., 1991), soon to be followed by other species, including the human H_1R (De Backer et al., 1993; Fukui et al., 1994; Moguilevsky et al., 1994). The human H_1R gene is an intron-less gene that is located on chromosome 3p25 and encodes for a 487 amino acid GPCR with a long third intracellular loop (IL3) (De Backer et al., 1998).

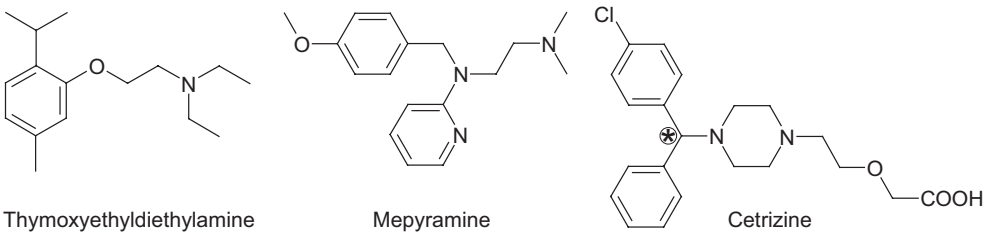

Figure 8. Chemical structures of H_1R ligands.

The H_1R predominantly couples to $G\alpha_{q/11}$-proteins (Leopoldt et al., 1997) leading to the activation of PLC and subsequent release of the second messengers IP_3 and DAG followed by the activation of PKC and the release of $[Ca^{2+}]_i$. Additionally, the H_1R has been shown to constitutively increase IP_3 levels (Bakker et al., 2000) and

activate the nuclear factor κ B (NF-κB) (Bakker et al., 2001), a transcription factor involved in inflammation and cancer. Remarkably, the H_1R-mediated constitutive activation of NF-κB is primarily mediated through βγ-subunits, whereas both $G\alpha_{q/11}$-proteins and βγ-subunits are required for the H_1R agonists mediated NF-κB activation (Bakker et al., 2001).

Histamine H_2 receptor

The histamine H_2 receptor (H_2R) are located in a variety of tissues including brain, gastric cells and cardiac tissue (Hill et al., 1997). H_2R are involved in the gastric acid secretion and therefore inverse agonists for the H_2R are used in the treatment of peptic ulcers. The first H_2R antagonist, burimamide (Black et al., 1972b), was not very potent and actually not very specific for the H_2R either. With the discovery of the H_3R and H_4R we now know that burimamide even has a higher affinity for the H_3R and H_4R (Arrang et al., 1983; Lim et al., 2005). Further development of H_2R antagonists led to the discovery of cimetidine (Tagamet®) by Smith, Kline & French and ranitidine (Zantac®) by Glaxo. These H_2R antagonists were widely used in the clinical treatment of peptic ulcers. However, nowadays is has become apparent that this condition is more effectively treated by a proton-pump inhibitor in combination with antibiotics when an infection with *H. pylori* is found (Deakin and Williams, 1992; Penston, 1996; Freston, 2000).

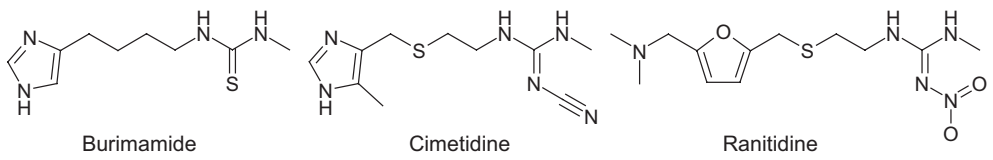

Figure 9. Chemical structures of H_2R ligands

The H_2R gene was the first of the histamine receptors to be cloned. By using degenerate oligonucleotide primers based on the known homology between GPCRs and subsequent polymerase chain reaction (PCR) on canine gastric parietal cell cDNA Gantz and coworkers cloned the canine H_2R (Gantz et al., 1991c). High homology of the various H_2R facilitated cloning of the H_2R in other species, including the human H_2R gene (Gantz et al., 1991a). The human H_2R gene is an intron-less gene located on chromosome 5q35 encoding for a protein of 358 amino acids. Compared to the other histamine receptors the H_2R has a short IL3 and a longer C-terminal tail. The H_2R predominantly couples to $G\alpha_s$-proteins and subsequently leads an increase in intracellular cAMP and the activation PKA. Selective immunoprecipitation of activated G proteins labeled with [α-^{32}P]GTP azidoanilide revealed, in addition to $G\alpha_s$-protein coupling, specific coupling to $G\alpha_q$-

proteins as well. Stimulation of recombinantly expressed H_2R in COS-7 cells indeed resulted in an increase in intracellular IP_3 and as well as an increase in cAMP (Kuhn et al., 1996). Similar to the H_1R, the H_2R was found to display constitutive activity as well (Smit et al., 1996), which led to the reclassification of heretofore know antagonists (like cimetidine and ranitidine) as inverse agonists. Burimamide was found to be neutral antagonist for the rat H_2R (Smit et al., 1996), but acted as a weak partial agonist on the human H_2R (Alewijnse et al., 1998).

Histamine H_3 receptor

The histamine H_3 receptor (H_3R) is predominantly expressed in the CNS, and to a lesser extent in the peripheral nervous system (Hill et al., 1997). Histaminergic neurons originate from the tuberomammillary nucleus in the posterior hypothalamus, which is also the sole location of HDC (Ericson et al., 1987), and send widespread projections to various brain regions (Lin, 2000; Haas and Panula, 2003). On histaminergic neurons in the CNS the H_3R acts as an presynaptic autoreceptor inhibiting the release and synthesis of histamine (Arrang et al., 1983). On non-histaminergic neurons in mammalian brain, the H_3R functions as a heteroreceptor inhibiting the release of various important neurotransmitters like serotonin, noradrenalin, acetylcholine, and dopamine (Hill et al., 1997). Besides neuronal expression, peripheral inhibitory effects of H_3R activation on neurotransmission have been shown to occur in the cardiovascular system, gastrointestinal tract, and the airways (Schwartz et al., 1990; Bertaccini and Coruzzi, 1995; Delaunois et al., 1995; Malinowska et al., 1998). It is currently not know if these H_3Rs are located on peripheral tissues themselves or on nerve terminals of neurons innervating these organs, as shown for H_3Rs on perivascular nerve terminals (Ishikawa and Sperelakis, 1987). In contrast, in adults H_3R mRNA appeared to be almost exclusively expressed in the CNS with high expression in basal ganglia, globus pallidus, hippocampus, and cortex (Martinez-Mir et al., 1990; Lovenberg et al., 1999; Karlstedt et al., 2003).

The H_3R is considered to be an attractive target for both academia and the pharmaceutical industry because of several reasons (Leurs and Timmerman, 1992; Alguacil and Perez-Garcia, 2003; Leurs et al., 2005; Hancock, 2006; Bonaventure et al., 2007). First of all, the H_3R is expressed in brain regions that are critical for cognition (cortex and hippocampus), sleep and homeostatic regulation (hypothalamus) (Hancock and Fox, 2004). Moreover, the H_3R act as a heteroreceptor modulating the release of several important neurotransmitters that are involved in processes like cognition, mood, and sensory gating (Schlicker et al., 1994b; Caulfield and Birdsall, 1998; Missale et al., 1998). In addition, the H_3R acts as an autoreceptor regulating the release and synthesis of histamine, a neurotransmitter that plays a role in vigilance, attention, impulsivity, and

feeding/weight regulation (Schwartz et al., 1991; Hill et al., 1997). Therefore, antagonists for the H_3R are currently under investigation in several therapeutic areas including sleep disorders, energy homeostasis and cognitive disorders (Passani et al., 2004; Wijtmans et al., 2007).

The first developed H_3R ligands, like thioperamide and clobenpropit (Arrang et al., 1987; van der Goot et al., 1992)., were based on the structure of histamine and are mostly imidazole-based (Stark et al., 2004). However, development of H_3R specific antagonists by pharmaceutical companies like, JNJ, Schering, Abbott, GSK, Pfizer, UCB Pharma, Merck, Banyu, Lilly, Sanofi-Synthelabo and Roche focused on non-imidazole compounds (Cowart et al., 2004; Wijtmans et al., 2007), because many imidazole containing compounds could potentially have drug-drug interactions as they interact with the cytochrome P450 isozymes (LaBella et al., 1992; Yang et al., 2002).

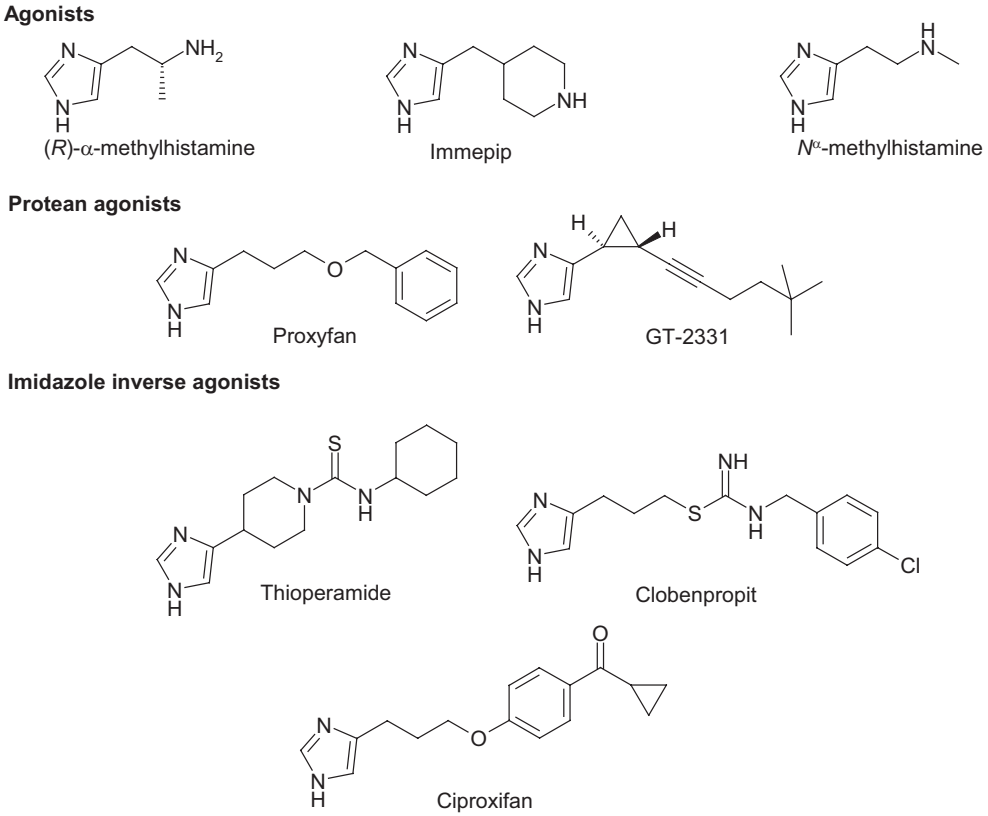

Figure 10. Chemical structures of imidazole-containing H_3R ligands.

Sleep disorders

The sedative effects of first generation H_1R antagonists like mepyramine already indicated a role for the histaminergic system in the sleep-wake cycle. Besides blockade of H_1Rs, an increase in brain histamine induced by α-fluoromethylhistidine, an irreversible inhibitor of histidine decarboxylase, leads to a decrease on the sleep-waking parameters and conversely a decrease of brain histamine levels by SKF-91488, a specific inhibitor of histamine-N-methyltransferase, increases wakefulness (Kiyono et al., 1985; Lin et al., 1988). Mice that lack the ability to synthesize histamine, $HDC^{-/-}$ mice, show an increase in paradoxical sleep and changes in the cortical-EEG. Moreover, when these mice were behaviorally and cognitively challenged they were unable to remain awake (Parmentier et al., 2002). More recently is was shown that histamine release in the cortex indeed strongly correlates with waking (Chu et al., 2004). Interestingly, Modafinil (Provigil®) currently on the market for the treatment of narcolepsy, obstructive sleep apnea/hypopnea and shift work sleep disorders, increases histamine levels in the hypothalamus and depletion of neuronal histamine by α-fluoromethylhistidine abolished the effect of modafinil on locomotor activity in mice (Ishizuka et al., 2003; Ishizuka et al., 2007).

The H_3R co-localizes with histaminergic neurons in brain regions that regulate the sleep-wake cycle and it controls the histamine levels through presynaptic auto-inhibition,. Therefore the H_3R is considered as a potential target for the control of arousal, vigilance and H_3R ligands are suggested to be beneficial in the treatment sleep disorders. Moreover, the histaminergic system is associated with the orexinergic neurons (Peyron et al., 1998; Huang et al., 2001; Ishizuka et al., 2002). This system is associated with food intake and wakefulness (Kilduff and Peyron, 2000), and orexin increases histamine release from the hypothalamus (Eriksson et al., 2001; Yamanaka et al., 2002).

Studies with H_3R agonists, such as SCH 50971, (*R*)-α-methylhistamine and its pro-drug BP 2-94 show an increase in slow wave sleep and a decrease in wakefulness and REM sleep (Lin et al., 1990; Monti et al., 1991; Monti et al., 1996; McLeod et al., 1998), whereas H_3R antagonists, such as thioperamide, JNJ-5207852, BF2.649 and ciproxifan, increase the time spent awake and decrease REM sleep and slow-wave sleep and have no such effects in $H_3^{-/-}$ mice (Lin et al., 1990; Monti et al., 1991; Barbier et al., 2004; Ligneau et al., 2007; Parmentier et al., 2007).

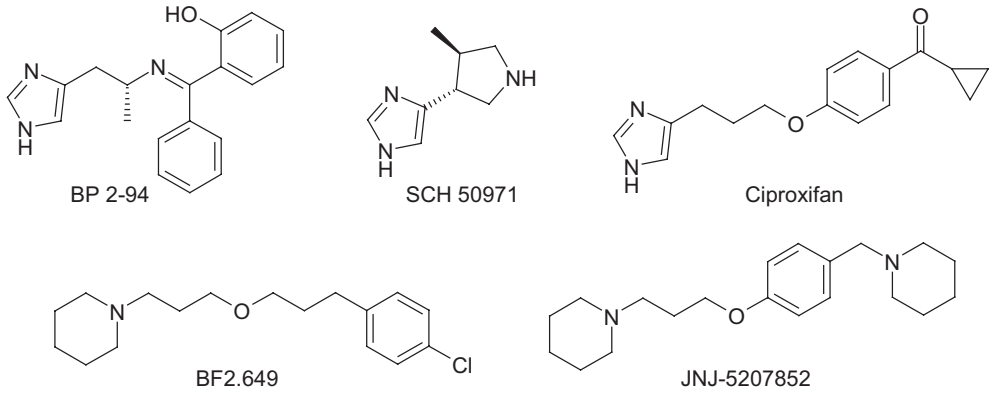

Figure 11. Chemical structures of H_3R ligands developed for sleep disorders.

Obesity

A number of studies indicate that histamine may play a critical role in food intake and changes in body weight (Sakata et al., 1991; Machidori et al., 1992; Tuomisto et al., 1994; Sakata, 1995; Sakata et al., 1997). Moreover, histamine containing neurons project to paraventricular nucleus and ventromedial hypothalamus (Schwartz et al., 1991; Onodera et al., 1994), regions know to be involved in the regulation of feeding behavior and body weight (King, 2006). H_1R antagonists were shown to increased food intake and conversely activation of brain H_1R decreased food intake in rats (Mercer et al., 1994; Lecklin et al., 1998). Moreover, the H_3R antagonist thioperamide was shown to reduce feeding in rats (Sakata et al., 1991; Itoh et al., 1998). The recent cloning of the H_3R (Lovenberg et al., 1999) has made it possible to create $H_3R^{-/-}$ mice. These mice are reported to have either no significant difference in body weight compared to wild-type mice (Toyota et al., 2002) or to have a mild obese phenotype that is characterized by an increase in body weight, food intake, adiposity and a reduction in energy expenditure (Takahashi et al., 2002). The obese phenotype of the $H_3R^{-/-}$ mice is actually in contrast with earlier work on histamine physiology, the effect H_1R compounds and H_3R antagonists. This apparent discrepancy could be due to an alter neuronal development in the absence of the H_3R or alterations in the post-synaptic histamine receptors (Tokita et al., 2006).

To evaluate the H_3R as a potential target against obesity various non-imidazole containing compounds were developed and evaluated. The Novo Nordisk compounds NNC 38-1049 and NNC 38-1202 reduce the body weight of normal and dietary obese rats and this is associated with a decrease in food intake and an increase in extracellular histamine concentrations in the hypothalamus (Malmlof et al., 2005; Malmlof et al., 2006). Furthermore, NNC 38-1202 was found to decrease plasma triglycerides paralleled with increases in plasma free fatty acids and beta-

hydroxybutyrate levels and to be effective in higher mammalian species like pig and obese rhesus monkeys (Malmlof et al., 2006; Malmlof et al., 2007). The non-imidazole H_3R compounds developed by Abbott Laboratories, A-331440 and its non-genotoxic analogue A-417022, were also shown to decrease obesity-related parameters in dietary-induced obese mice (Hancock et al., 2004a; Hancock et al., 2004b; Hancock et al., 2005).

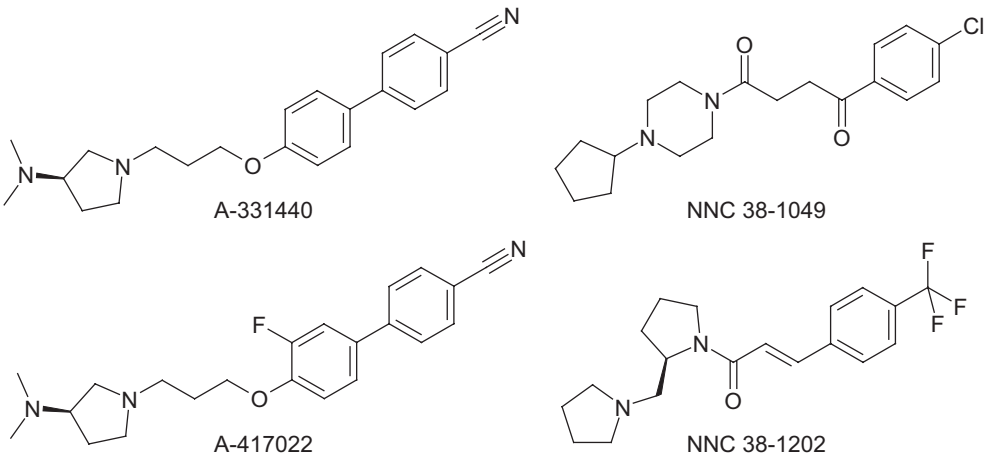

Figure 12. Chemical structures of H_3R ligands developed against obesity.

Cognitive disorders

Histaminergic neurons arising from the posterior hypothalumus project to brain areas important for cognition and emotion like the cerebral cortex, amygdala, thalamus and basal forebrain (Panula et al., 1984; Watanabe et al., 1984). Neuronal histamine has been implicated in various brain functions including learning and memory (Brown et al., 2001; Haas and Panula, 2003). For example, intracerebroventricular administration of histamine was shown to facilitate memory (de Almeida and Izquierdo, 1986; Kamei and Tasaka, 1993), while its depletion impairs the acquisition of active avoidance response (Kamei et al., 1993).

Conversely, a negative influence of neuronal histamine on learning and memory has also been suggested. HDC knockout mice showed improved water-maze performance during both hidden and cued platform tasks, but were deficient in object discrimination, indicating that brain histamine synthesis can have both memory promoting and suppressive effects via distinct and independent mechanisms (Dere et al., 2003). Similarly, bilateral electrolytic or neurotoxic lesion of the tuberomammillary nuclei was found to facilitate the performance of rats in a variety of learning tasks (Huston et al., 1997).

Pharmacological intervention of the histamine receptors supports the role of histamine in cognition. H_1R antagonists were shown to deteriorate the cognitive performance in attention-demanding task while there was no significant change in subjective sleepiness in the same dose (Okamura et al., 2000). In this regards, the H_3R generates the most interests of the histamine receptors as a therapeutic target for cognitive diseases like attention-deficit hyperactivity disorder (ADHD), symptomatic treatment of dementia in Alzheimer's disease and schizophrenia. Many of the neurotransmitters that are regulated by the H_3R have been shown to be altered in Alzheimer's disease (Nordberg, 1992; Storga et al., 1996), including histamine (Airaksinen et al., 1991; Schneider et al., 1997; Panula et al., 1998). Moreover, H_3R in medial temporal cortex are preserved in late-stage of Alzheimer's disease (Medhurst et al., 2007).

The effects of H_3R agonists on cognition have been related to modifications of acetylcholine neurotransmission (Arrang et al., 1995; Blandina et al., 1996). H_3R agonist induced memory impairments for object recognition and passive avoidance in rats was associated with inhibition of cortical acetylcholine release (Blandina et al., 1996), a neurotransmitter linked to cognitive function (Phillis, 2005). The effect of the H_3R on neuronal acetylcholine is indirect (Arrang et al., 1995; Blandina et al., 1996) and depends on GABAergic neurons (Giorgetti et al., 1997). Post-training administration of H_3R agonists did not lead to a memory impairment, suggesting that the H_3R is involved in the acquisition rather than memory recall (Giovannini et al., 1999). Moreover, H_3R-mediated acetylcholine release in the amygdala and hippocampus was shown to be important for the consolidation of fear memory in rats (Passani et al., 2001; Cangioli et al., 2002). In contrast, H_3R agonists were shown to beneficial for spatial learning and reduced the scopolamine-induced memory deficit in rats assessed in a water maze (Smith et al., 1994).

Conversely, H_3R antagonists were shown to reverse a scopolamine-induced deficit in a rat two-choice discrimination water maze, but these effects were less pronounced in the mouse Barnes maze (Komater et al., 2005), indicating that inherent differences in behavioral tests or species differences might lead to different outcomes in these behavioral experiments. Moreover, different behavioral tests to assess memory might involve different brain areas. Some reports show a beneficial effect of H_3R antagonists on memory (Prast et al., 1996). In contrast, other studies only show a pro-cognitive effect in the presence of a deficit like a scopolamine induced amnesia or in senescence-accelerated mice (Giovannini et al., 1999), an effect that might be dependent on H_1Rs (Miyazaki et al., 1995; Miyazaki et al., 1997). More recently, in a repeated acquisition version of an inhibitory avoidance task using spontaneously hypertensive rats H_3R antagonists were shown to enhance performance (Fox et al., 2002).

With the discovery of new highly potent and selective non-imidazole H3R antagonists it is becoming more clear the inhibition of the H3R might improve cognitive performance in e.g. symptomatic treatment of dementia in Alzheimer's disease, schizophrenia and other cognitive disorders (Esbenshade et al., 2006a). In a five-trial rat pup avoidance test A-304121, A-317920, A-349821 and ABT-239 improved cognitive performance and social memory comparable to previously published observations for imidazole H_3R antagonists (Cowart et al., 2005; Esbenshade et al., 2005; Fox et al., 2005). ABT-239 was also tested in several schizophrenia models and shown to improved gating deficits in mice using pre-pulse inhibition of startle and in N40 gating studies. Furthermore, ABT-239 attenuated methamphetamine-induced hyperactivity in mice, enhanced acetylcholine release in adult rat frontal cortex and hippocampus and enhanced dopamine release in frontal cortex, but not striatum (Ligneau et al., 2007). Another non-imidazole compound, BF2.649, was shown to be beneficial in a two-trial object recognition test in mice, in both a scopolamine-induced and a natural memory loss paradigm (Medhurst et al., 2007). Also GSK189254 was shown to significantly improved performance of rats in diverse cognition paradigms, like passive avoidance, water maze, object recognition and attentional set shift. Moreover, GSK189254 increased the release of acetylcholine, noradrenalin, and dopamine in the anterior cingulate cortex and acetylcholine in the dorsal hippocampus. Additionally, c-Fos immunoreactivity was found to be increase after treatment with GSK189254 in the prefrontal and somatosensory cortex (Hancock et al., 2006). The increased c-Fos immunoreactivity after treatment with H_3R inverse agonists in cingulate cortex and hippocampus has been suggested to be indicative for the procognitive effect of H_3R compounds, as H_3R inverse agonists suggested to be beneficial for the treatment of obesity, like A-331440, did not increase c-Fos in these regions (Sumner et al., 2004). Expression of c-Fos has been used pre-clinically to study regions of neuronal activation resulting from psychopharmacological challenge and differential expression patterns of c-Fos are suggested to be predictive of therapeutic responses (de Esch et al., 2005).

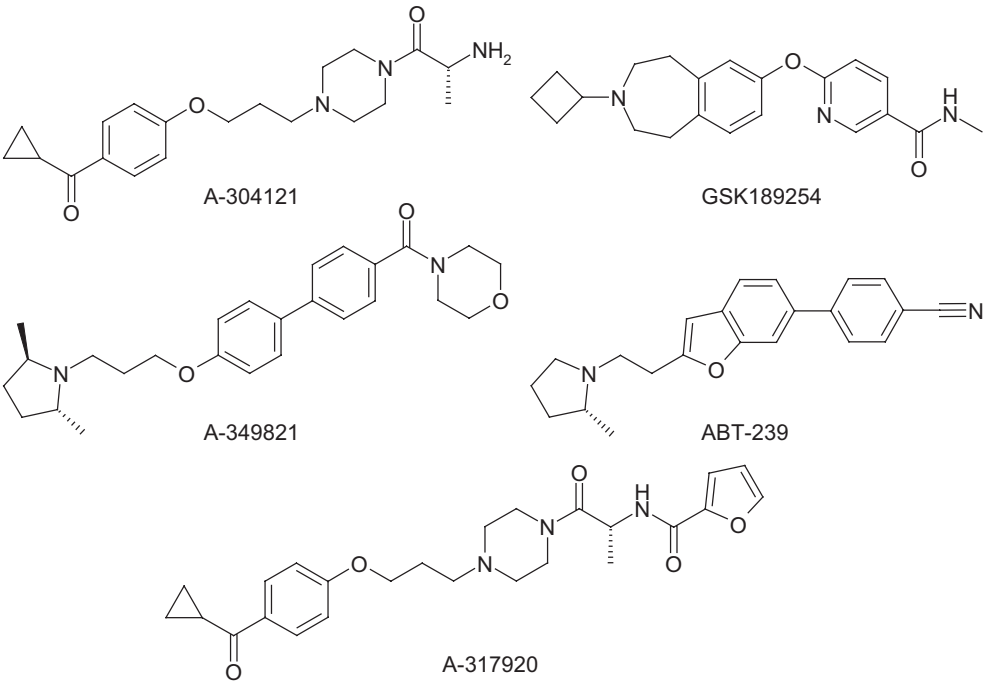

Figure 13. Chemical structures of H_3R ligands developed for cognitive disorders.

Therapeutic potential of the H_3R

As described, there is a wealth of knowledge known on both *in vitro* and *in vivo* data on the H_3R, establishing it as an attractive drug target. This is nicely illustrated by the steady increase in the number of H_3R-related PubMed entries and patents filled for the compounds targeting the H_3R, however no clinical data is available yet (Celanire et al., 2005).

The Gliatech compound, GT-2331 (Perceptin®), was the first H_3R compound to enter clinical trails, but studies regarding its potential to alleviate symptoms of ADHD were halted in phase II. Currently, several pharmaceutical companies have subjected H_3R compounds to pre-clinical studies and since 2003 two Abbott H_3R antagonists, ABT-239 and ABT 834, entered phase I studies for the treatment of ADHD and cognitive disorders respectively. In 2005 a Johnson & Johnson and a Glaxo-SmithKline H_3R antagonists entered phase I studies for the treatment of narcolepsy and dementia respectively (Celanire et al., 2005; Wijtmans et al., 2007) (Table 1).

Table 1. Patent applications and clinical status of claimed or identified imidazole and non-imidazole H_3R antagonists.

Company	New Chemical Entities	Clinical status	Therapeutic Indication
AstraZeneca	H_3/H_4 antagonists	preclinical	COPD, asthma
Abbott	ABT-834	Phase I (07/2003)	ADHD
	ABT-239	Phase I (11/2003)	Cognitive disorders
	A-431404	preclinical	
	A-424835	preclinical	
	A-349821	preclinical	
	A-320436	preclinical	
	A-331440	preclinical	Obesity
	A-417022	preclinical	
	A-423579	preclinical	
Banyu Pharm.	-	preclinical	CNS/peripheral. disorders
Bioprojet	BF-2649	Phase II	CNS disorders
Eli Lilly	-	preclinical	CNS disorders, obesity
Gliatech/Merck	GT-2331	Phase II halted	ADHD
	GT-2227	preclinical	
	GT-2394	preclinical	Obesity
Glaxo-SmithKline	GSK-189254A	Phase I (03/2005)	Dementia/nacrolepsy
	GSK-207040A	preclinical	CNS disorders
James Black Foundation	JB 98064 (NI)	preclinical	CNS/peripheral disorders
Johnson&Johnson	JNJ-17216498	Phase II	Narcolepsy
-Ortho McNeil	JNJ-5207852	preclinical	Narcolepsy
-Janssen Pharm.	JNJ-7737782	preclinical	
	JNJ-10181457	preclinical	CNS disorders
Merck	MK-0249	Phase II	CNS disorders
Novo-Nordisk	NNC-0038-1049 (NI)	preclinical	Obesity
Boehringer Ingelheim	NNC-0038-1202 (NI)	preclinical	
Sanofi-Aventis	-	preclinical	CNS/peripheral disorders
Schering-Plough	SCH-79687 (NI)	preclinical	Allergic rhinitis
Servier	-	preclinical	CNS disorders

Other aspects of the H_3R.

Aspects of the H_3R like genomic organization signal transduction, dimerization and characteristics of the H_3R isoforms will be discussed in more detail in chapter 2.

Histamine H_4 receptor

The H_4R has a low CNS expression and is mainly located in peripheral blood leukocytes and mast cells, suggesting a role for the H_4R in inflammatory and immune response. To establish the H_4R as a target for treating inflammatory conditions, potent and selective H_4R ligands were needed. However, many imidazole-containing H_3R ligands show a high affinity for the H_4R as well, which is probably due to a homology of 68% transmembrane region with the H_3R (Liu et al., 2001a; Liu et al., 2001b; Nguyen et al., 2001; Lim et al., 2005). Classical H_3R ligands like the H_3R agonists immepip and imetit, and the H_3R inverse agonist clobenpropit were shown to be potent high affinity agonists on the H_4R (Jablonowski et al., 2003), whereas thioperamide was found to be a high affinity inverse agonists for the H_4R (Lim et al., 2005). The first potent and H_4R selective agonists, 4-methylhistamine (Lim et al., 2006) and VUF8430 (Jablonowski et al., 2003) and inverse agonists, JNJ 7777120 (Terzioglu et al., 2004) and its benzimidazole derivative VUF6002 (Esbenshade et al., 2004), have now been developed. These specific H_4R ligands, together with the development of H_3R specific ligands (de Esch et al., 2005), will help to delineate the roles of the H_4R *in vivo*.

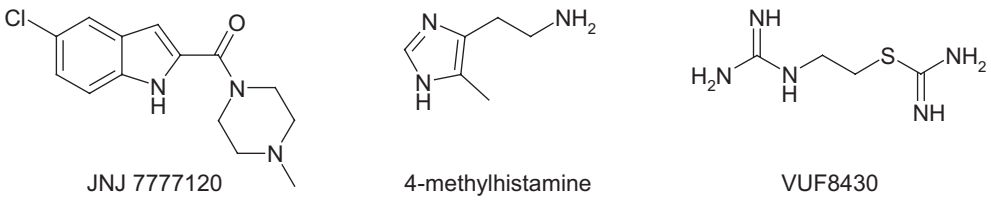

Figure 14. Chemical structures of H_4R ligands.

The gene that encodes for the human histamine H_4 receptor (H_4R) is located on chromosome 18q11.2 and contains three exons encoding for a 390 amino acid protein that has a 31% homology to the human H_3R (Coge et al., 2001). Similarity in gene organization between the H_3R and the H_4R might indicate the possibility of H_4R isoforms, however so far no H_4R isoforms have been published (Nakamura et al., 2000; Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). Like the H_3R, the H_4R couples to $G\alpha_{i/o}$-proteins, subsequently leading to an inhibition of cAMP accumulation and the subsequent PKA dependent inhibition of the cAMP responsive element-binding protein (CREB) (Morse et al., 2001). Furthermore, activation H_4R has been shown to lead to a $G\alpha_{i/o}$-protein dependent phosphorylation of MAPK in HEK293 cells (Hofstra et al., 2003; Nakayama et al., 2004) and mobilization of $[Ca^{2+}]_i$ in mast cells endogenously expressing the H_4R mast cells and in L1.2 cells that recombinantly express the H_4R (Hofstra et al.,

2003). The H_4R mediated mobilization of $[Ca^{2+}]_i$ in mast cells is both $G\alpha_{i/o}$-protein and PLC dependent as shown by the use of PTX and the phospholipase C inhibitor U73122 (Lovenberg et al., 1999).

Chapter 2

Molecular aspects of the histamine H_3 receptor

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Abstract

The cloning of the histamine H_3 receptor (H_3R) cDNA in 1999 by Lovenberg et al. allowed detailed studies of its molecular aspects and indicated that the H_3R can activate several signal transduction pathways including $G_{i/o}$-dependent inhibition of adenylyl cyclase, activation of phospholipase A_2, Akt and the mitogen activated kinase as well as the inhibition of the Na^+/H^+ exchanger and inhibition of K^+-induced Ca^{2+} mobilization. Moreover, cloning of the H_3R has led to the discovery several H_3R isoforms generated through alternative splicing of the H_3R mRNA. The H_3R has gained the interest of many pharmaceutical companies as a potential drug target for the treatment of various important disorders like obesity, myocardial ischemia, migraine, inflammatory diseases and several CNS disorders like Alzheimer's disease, attention-deficit hyperactivity disorder and schizophrenia. In this paper we review various molecular aspects of the hH_3R including its signal transduction, dimerization and the occurrence of different H_3R isoforms.

Introduction

In the historical context of histamine's pharmacology our current knowledge on the third histamine receptor has been gathered in a very short period of time. After the discovery of histamine's biological actions in 1910 (Dale and Laidlaw, 1910b), the first two histamine receptors were proposed in 1966 (Ash and Schild) and 1972 (Black), based on classical pharmacological rules of drug selectivity (Ash and Schild, 1966; Black et al., 1972a). Using a similar strategy it was ultimately the French research group at INSERM, led by Jean-Michel Arrang and Jean-Charles Schwartz, which described in 1983 for the first time an additional histamine receptor, mediating a negative feedback on the release of histamine from rat brain slices (Arrang et al., 1983).

With the rapid expansion in the knowledge on the molecular aspects of the histamine H_3 receptor (H_3R) following cloning of the receptor cDNA, it has been recognized as a promising G-protein coupled receptor (GPCR) target in the CNS for the treatment of a variety of diseases, e.g. obesity and cognitive disorders (for detailed reviews see (Hancock and Fox, 2004; Celanire et al., 2005; Hancock and Brune, 2005; Leurs et al., 2005; Esbenshade et al., 2006a)). Moreover, at present we are overwhelmed with a large increase in our knowledge on the molecular aspects of H_3R. Especially in the last decade important new data have been generated, following the seminal paper of the Johnson & Johnson team lead by Tim Lovenberg on the cloning of the human H_3R (hH_3R) (Lovenberg et al., 1999). Despite the fact that both the histamine H_1 and H_2 receptor cDNA's sequences were known since the early nineties (Gantz et al., 1991b; Yamashita et al., 1991)

and substantial efforts of various laboratories to clone the H_3R cDNA on the basis of homology with the other two histamine receptors, it lasted until 1999 to elucidate the molecular architecture of the hH_3R (Lovenberg et al., 1999). Following a large scale effort to clone CNS-expressed (orphan) GPCRs, Lovenberg and colleagues identified and subsequently 'deorphanised' the hH_3R (Lovenberg et al., 1999). The isolated hH_3R cDNA encoded a 445 amino acid protein with all the hallmarks of the family A, rhodopsin-like GPCR (Leurs et al., 2000), and finally confirmed initial suggestions of the GPCR nature of the H_3R based on H_3R agonist-induced [^{35}S]GTPγS binding (Clark and Hill, 1996; Laitinen and Jokinen, 1998), GTP- and PTX-sensitivity of H_3R radioligand binding and/or responses (Clark et al., 1993; Jansen et al., 1994; Clark and Hill, 1996).

With the identification of the hH_3R cDNA, histamine receptor research was boosted a great deal and enormous progress has been made in the field ever since. The new information resulted in the identification of a novel histamine receptor, H_4 (Oda et al., 2000), and also evoked strong interest of many pharmaceutical companies to develop H_4R selective ligands (Celanire et al., 2005; Leurs et al., 2005). Whereas the H_3R has been considered by many companies as an interesting target even before 1999, the lack of molecular information and thus the availability of recombinant systems, made most companies hesitant to start drug discovery programs. A recent review by Art Hancock on the large drug discovery efforts by Abbott Laboratories, nicely illustrates how the lack of the hH_3R as a screening tool resulted in an initial setback in Abbott's H_3R program (Hancock, 2006). Nevertheless, their early entry in the H_3R field ensured Abbott a strong position in the present H_3R field (Celanire et al., 2005; Hancock, 2006). With the present availability of the H_3R cDNA many major pharmaceutical companies have joined the search for selective and potent H_3R antagonists (Celanire et al., 2005). The development of H_3R ligands has recently been elaborately documented in various reviews (Cowart et al., 2004; Stark et al., 2004; Celanire et al., 2005; Esbenshade et al., 2006a; Hancock, 2006).

The cloning of the H_3R cDNA has also led to a detailed delineation of several molecular aspects of H_3R pharmacology. With the identification of the chromosomal localization and the elucidation of the genomic H_3R sequence, it became clear that the H_3R gene contains various introns and, thus, alternative splicing might result in various H_3R isoforms. Indeed, soon after the cloning of the hH_3R cDNA, at least 20 human (Nakamura et al., 2000; Cogé et al., 2001; Tardivel-Lacombe et al., 2001; Tsui, 2001a; Tsui, 2001b; Wellendorph et al., 2002; Gallagher and Yates, 2003) and several rodent (Drutel et al., 2001; Morisset et al., 2001) isoforms have been identified. In this review we present an overview of the H_3R isoforms and their known signal transduction pathways for a better

understanding of the mechanism of action of H_3R antagonists as potential therapeutics.

Genomic organization of the H_3R

The hH_3R gene is located on chromosome 20 at location 20q13.33 (HRH3 GeneID: 11255) and the coding region has been suggested to consist of either three exons and two introns (GenBank Accession number AL078633) (Wiedemann et al., 2002), or four exons and three introns (Cogé et al., 2001). Alternatively, the most 3' intron has been proposed to be a pseudo-intron as it is retained in the $hH_3R(445)$ isoform, but deleted in the $hH_3R(413)$ isoform (Tardivel-Lacombe et al., 2001). In the coding region for the $hH_3R(445)$ exon 1 codes for transmembrane domain (TM) 1 and half of TM2, exon 2 codes for half of TM2 and TM3 and exon 3 encodes the remaining TM domains (Figure 1). The complete coding sequence spans almost 4 kbp (nt 15421-19670). As reviewed extensively elsewhere (Hancock et al., 2003; Bakker, 2004; Leurs et al., 2005), soon after the cloning of the hH_3R gene, the highly conserved H_3R genes were cloned by sequence homology from various other species, including rats (Lovenberg et al., 2000; Drutel et al., 2001; Morisset et al., 2001; Wulff et al., 2002) guinea-pigs (Cassar, 2000; Tardivel-Lacombe et al., 2000), mice (Chen et al., 2003), and monkeys (Yao et al., 2003).

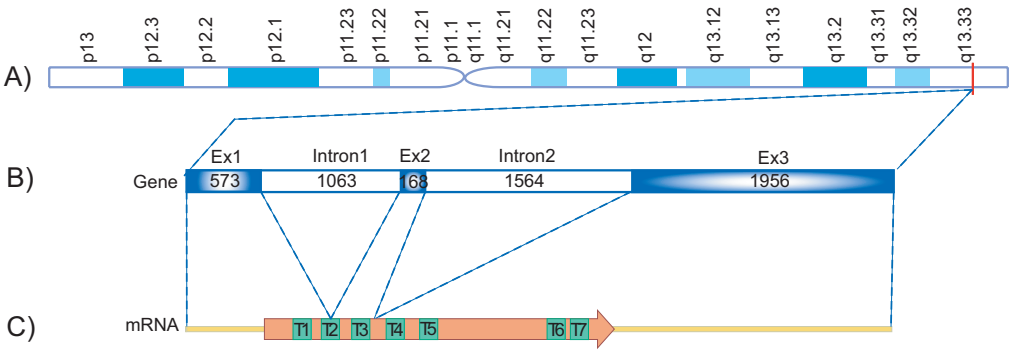

Figure 1. Genomic organization of the hH_3R. A) Schematic representation of the human chromosome 20 and the location of the hH_3R gene in the q13.33 region. B) Schematic representation of the hH_3R gene and its exons (dark blue) and introns (white boxes). C) Schematic representation of the H_3R mRNA showing the untranslated region (in yellow), the coding regions (orange) and the transmembrane domains (green).

Identification of H_3R isoforms

To date at least 20 isoforms of the hH_3R are known and in addition several H_3R isoforms have been identified in rat, guinea-pig and mouse as well (Nakamura et

al., 2000; Tardivel-Lacombe et al., 2000; Cogé et al., 2001; Drutel et al., 2001; Morisset et al., 2001; Tardivel-Lacombe et al., 2001; Wellendorph et al., 2002; Wiedemann et al., 2002; Rouleau et al., 2004; Ding et al., 2005). So far no isoforms were found for the monkey H_3R (Yao et al., 2003). The complete spectrum of H_3R isoforms might be highly species-specific, complicating the evaluation of the various isoforms in relation to the effectiveness of H_3R ligands in vivo.

For the hH_3R, alternative splicing occurs in four different regions. In three of these regions; 7-42, 85-98 and 197-417 (following the amino acid numbering of the $hH_3R(445)$ isoform), this leads to a deletion of various amino acids. In the fourth region, alternative splicing generates isoforms that have eight additional amino acids at the C-terminus, consequently adding the amino acids KMKKKTCL to the hH_3R protein. The third region (197-417), contains several donor and acceptors sites making it a highly diverse region. Currently, nothing is known about the regulation of the splicing of the H_3R mRNA. Since alternative splicing can occur simultaneously in the different indicated regions a large variety of different H_3R isoforms can be generated (Table 1 and Figure 2, 3).

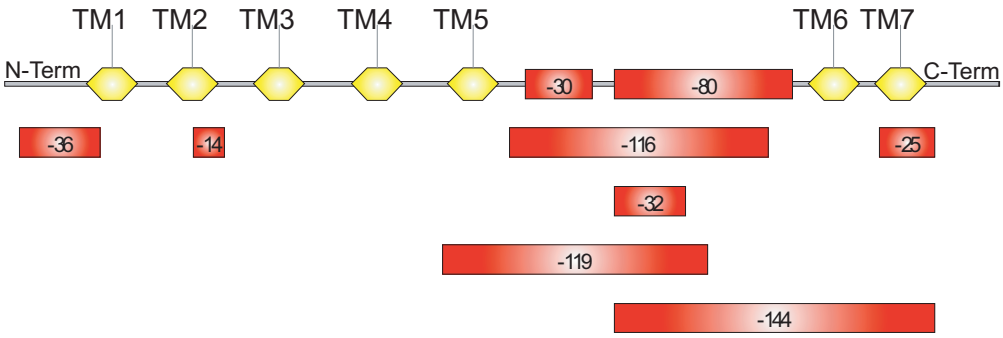

Figure 2. Schematic representation of the hH_3R protein showing the transmembrane domains (yellow) and the corresponding alternative splicing events, the numbering shows the number of deleted amino acids.

Alternative splicing in the first region deletes a part of the N-terminal tail and a part of TM1, whereas splicing in the second region deletes a part of the TM2. Alternative splicing in the third region between 226 and 353 generates hH_3R isoforms with a variation in the length of the third intracellular loop. Splicing in the third region, starting at amino acid 197 or ending at amino acid 417, leads to deletion of TM5 or TM6/7 respectively.

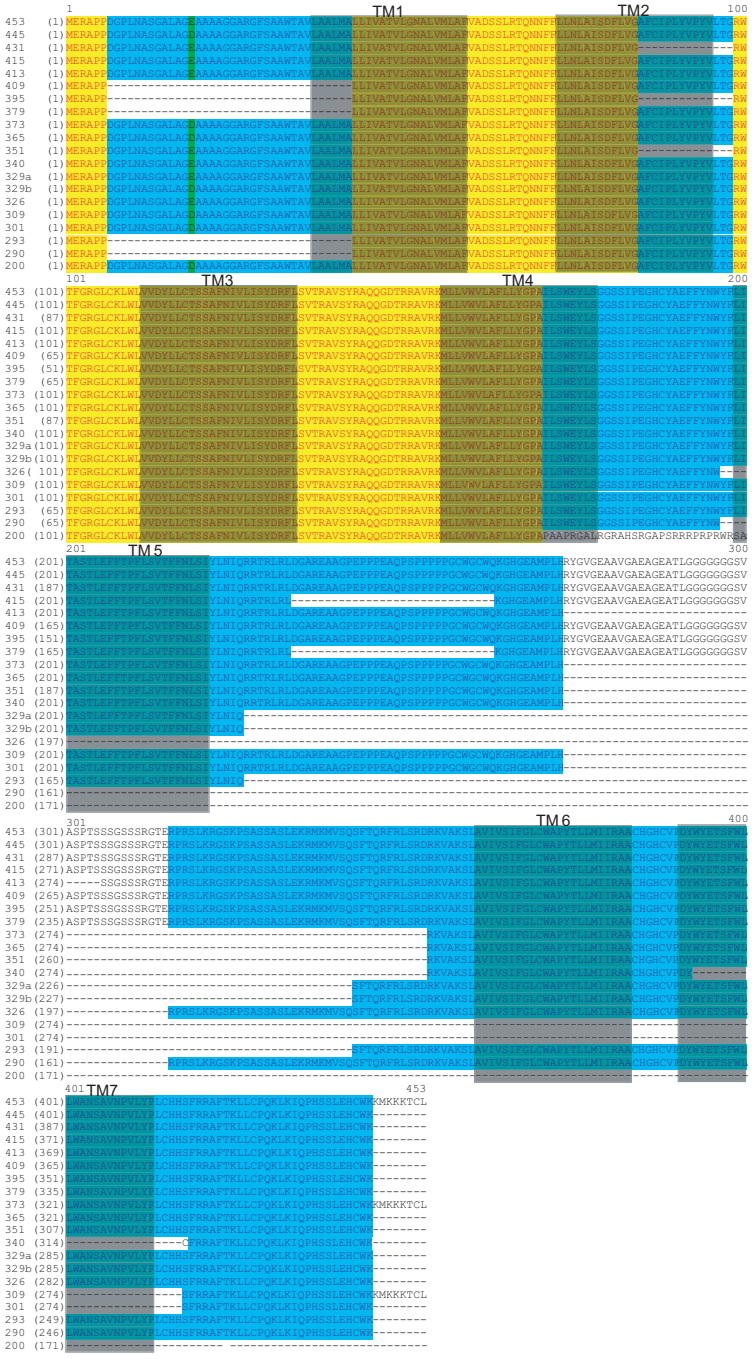

Figure 3. Alignment of the currently know hH_3R isoforms. The overlapping regions are in blue, partly overlapping regions yellow and the transmembrane domains are shaded black.

Following the cloning of the $hH_3R(445)$ by Lovenberg et al. (Lovenberg et al., 1999), Cogé et al. described the discovery of five additional isoforms with splicing in regions the between 85-98 or 197-353 ($hH_3R(431)$, $hH_3R(415)$, $hH_3R(365)$, $hH_3R(329)$ and $hH_3R(326)$ (Cogé et al., 2001)). In addition to confirming the $hH_3R(365)$ isoform, Wellendorph et al. described the cloning of four additional isoforms ($hH_3R(373)$, $hH_3R(309)$, $hH_3R(301)$ and $hH_3R(200)$ (Wellendorph et al., 2002)). The $hH_3R(373)$ is derived from the same splice event as the $hH_3R(365)$, but with eight additional amino acids at the C-terminus. The alternative splicing event leading to the addition of eight amino acids was also found to occur for the $hH_3R(445)$, leading to the $hH_3R(453)$ isoform (Nakamura et al., 2000). The $hH_3R(301)$ and the $hH_3R(309)$ are generated by splicing between amino acids 274 and 417, with or without the eight additional amino acids at the C-terminus. The $hH_3R(200)$ is created by a frame shift leading to a novel stop codon. Furthermore, Tradivel-Lacombe et al. described alternative splicing event to occur between 274 and 305 leading to the $hH_3R(413)$ isoform. Besides the scientific literature, information about H_3R isoforms can also found in the patent literature. Patent WO/2003/042359 by Merck claims twelve isoforms, including all isoforms previously published by Cogé et al. (Cogé et al., 2001) and six isoforms that were not known before ($hH_3R(409)$, $hH_3R(395)$, $hH_3R(379)$, $hH_3R(329b)$, $hH_3R(293)$, $hH_3R(290)$ (Gallagher and Yates, 2003)). All these isoforms are formed after deletion of amino acids 7-42 and five of them are derived through a combination of already known splice events in the 197-353 region. Patents by SmithKline Beecham describe splicing events leading to the $hH_3R(365)$ in combination with amino acid deletion the 85-98 or 393-417 region (Tsui, 2001a; Tsui, 2001b). Besides the already known isoforms, one can envision that by combination of the known splice sites more possibilities for hH_3R isoforms exist.

Table 1. Overview of human H_3R isoforms. The H_3R isoforms are denoted by their number of amino acids. Indicated are the regions of alternative splicing and the location in the protein. H_3R isoforms were shown to have radioligand binding (B), to be functional (F), or non-functional (NF). For most isoforms neither was determined (ND). Adapted from Leurs et al. 2005 (Leurs et al., 2005).

H_3R(aa)	A 7-42	B 85-98	197	226	234	263	C (variable area) 274	305	315	341	353	393	417	D	Functional	First Reference
453														+8	B/F	[28]
445															B/F	[10]
431		-14													NB	[22]
415					-30										ND	[22]
413							-32								ND	[23]
409	-36														B	[27]
395	-36	-14													ND	[27]
379	-36				-30										ND	[27]
373							-80							+8	F	[24]
365							-80								B/F	[22]
351		-14					-80								ND	[25]
340							-80					-25			ND	[26]
329a				-116											B	[22]
329b	-36						-80								ND	[27]
326			-119												ND	[22]
309							-144							+8	ND	[24]
301							-144								NF	[24]
293	-36			-116											ND	[27]
290	-36		-119												ND	[27]
200	spliced at aa 170 + 30 new aa														NF	[24]
Location	N-Term	TM2	TM5				IL3					TM6	TM7	C-Term		

H_3R Signal transduction

Inhibition of adenylyl cyclase

Early experiments studying the receptor function employing pertussis toxin (PTX) using various assay systems, such as AtT-20 cells endogenously expressing the H_3R (Clark et al., 1993), the guinea pig atria (Endou et al., 1994) and modulation of the H_3R induced [^{35}S]-GTPγS binding in rat brain (Clark and Hill, 1995), suggested that the H_3R might be G$\alpha_{i/o}$-coupled (Clark and Hill, 1996). Expression of the cloned H_3R cDNA in SK-N-MC cells confirmed the linkage of the hH_3R to G$\alpha_{i/o}$-proteins by showing its ability to inhibit the forskolin induced cAMP formation in a PTX sensitive manner (Figure 4) (Wieland et al., 2001). Subsequently, the hH_3R was shown to couple negatively to adenylyl cyclase in variety of heterologously transfected cell lines (Morisset et al., 2000; Cogé et al., 2001; Wieland et al., 2001; Gomez-Ramirez et al., 2002; Uveges et al., 2002) and in rat striatal slices (Sanchez-Lemus and Arias-Montano, 2004). Inhibition of adenylyl cyclase by the H_3R causes a decrease in intracellular cAMP and a subsequent reduction of protein kinase A (PKA) activity. PKA participates in a variety of signaling pathways leading to a range of biological responses including gene expression, synaptic plasticity, and behavior (Brandon et al., 1997). Some controversy exists on the role of cAMP in the modulation of neurotransmitter release by the presynaptic H_3R.

H_3R-mediated inhibition of cholinergic neurotransmission in the guinea pig ileum and the release of norepinephrine from mouse cortex was shown to be independent of adenylyl cyclase (Poli et al., 1993; Schlicker et al., 1994a; Lee and Parsons, 2000). However, more recent studies have shown that the H3R modulates synthesis of histamine (Gomez-Ramirez et al., 2002; Torrent et al., 2005; Moreno-Delgado et al., 2006b), as well as the exocytosis of norepinephrine both in cardiac synaptosomes, and in a transfected cell line (Seyedi et al., 2005) depends on the H_3R-mediated inhibition of cAMP levels.
Like many other GPCRs (Costa and Cotecchia, 2005; Bond and Ijzerman, 2006), the H_3R can be spontaneously active in the absence of histamine (Morisset et al., 2000; Wieland et al., 2001). This constitutive activity was demonstrated by the activation of the adenylyl cyclase pathway in CHO cells, in which enhanced receptor expression of either the rat H_3R(445) or H_3R(413) was associated with an increase in the constitutive inhibition of adenylyl cyclase. Many classical H_3R antagonists (e.g. thioperamide, clobenpropit, ciproxyfan) were shown to reverse this constitutive inhibition of adenylyl cyclase in stably transfected CHO (Morisset et al., 2000), SK-N-MC (Wieland et al., 2001) and HEK293 (Wulff et al., 2002) cells and, thus, are in fact H_3R inverse agonists. Besides agonists and inverse agonists, also neutral H_3R antagonists for the adenylyl cyclase pathway have been found. N-isopropylimpentamine and a propylene analogue of immepip, VUF5681, did not affect constitutive signaling, but these neutral H_3R antagonists competitively blocked the effect of H_3R agonists and H_3R inverse agonists (Wieland et al., 2001; Kitbunnadaj et al., 2003). Recently VUF5681 was used in vivo and was shown to block the effects of H_3R inverse agonist thioperamide on PKA-mediated synthesis of histamine in rat brain cortex, whereas it did not modulate histamine synthesis, which would be indicative for a H_3R agonist (Moreno-Delgado et al., 2006b).

Activation of phospholipase A_2

Also the H_3R-mediated activation of phospholipase A_2 (PLA_2), leading to the release of arachidonic acid depends on the activation of $G\alpha_{i/o}$-proteins (Figure 4). The release of arachidonic acid has been suggested to be important in the H_3R-mediated relaxation of the guinea pig epithelium (Burgaud and Oudart, 1993). PLA_2 activity is under the control of the high constitutive activity of the H_3R (Morisset et al., 2000). In CHO cells with moderate H_3R expression, proxyfan was shown to be a neutral H_3R antagonist in the [^{3}H]arachidonic acid release assay, but in CHO cells with high H_3R expression it displayed partial inverse agonism at the H_3R (Morisset et al., 2000). In contrast, proxyfan displayed partial H_3R agonism in a MAPK, [^{35}S]GTPγS and cAMP assays (Gbahou et al., 2003) and was therefore identified as a protean H_3R agonist, a ligand that depending on the system parameters, not on the receptor, displays distinct functional efficacy (Kenakin,

1995). In general, activation of PLA_2 not only leads to the release of arachidonic acid, but also to the release of docosahexaenoic acid and lysophospholipids. Besides having intrinsic physiological effects, these metabolites are also substrates for the synthesis of more potent lipid mediators such as platelet activating factor, eicosanoids, and 4-hydroxynonenal. The latter is the most cytotoxic metabolite, is associated with the apoptotic type of neural cell death and markedly increased in neurological diseases like ischemia, Alzheimer's disease and Parkinson's disease (Farooqui and Horrocks, 2006).

Modulation of the MAPK pathway

Besides H_3R-mediated signaling through $G\alpha_i$-subunits (Figure 4), $G\beta\gamma$-subunits are known to activate specific signal transduction pathways such as the MAPK pathway (Gutkind, 2000; Luttrell and Luttrell, 2003). MAPKs are known to have pronounced effects on cellular growth, differentiation and survival as well as to be important in neuronal plasticity and memory processes (Thiels and Klann, 2001; Thomas and Huganir, 2004). Activation of the rat H_3R was shown to lead to phosphorylation of MAPK in COS-7 cells heterologously expressing the rat H_3R. However, the level of phosphorylation varies for the different isoforms (Drutel et al., 2001). In contrast to the cAMP response, the H_3R was shown to exhibit little constitutive activation of the MAPK pathway (Gbahou et al., 2003). Whether this MAPK phosphorylation is solely due to $G\beta\gamma$-subunits, crosstalk with growth factor receptors or the use of scaffolds like β-arrestin (Lefkowitz and Shenoy, 2005), remains to be elucidated. Alternatively, a neuron-specific cascade from cAMP/PKA to MAPK comprising the critical events of hippocampus-based long-term plasticity has been described (Waltereit and Weller, 2003), but this pathway has yet not been studied in relation to the H_3R.

Activation of the Akt/GSK-3β axis

Also Akt/GSK-3β kinases have been shown to be activated by the H_3R in a neuroblastoma cell line, primary cultures of cortical neurons and in striatal slices of Spraque-Dawley rats (Figure 4) (Bongers et al., 2006). Like in the previously described pathways, the H_3R constitutively activates the Akt/GSK-3β axis, which can be reversed by the H_3R inverse agonist thioperamide. The H_3R-mediated activation Akt/GSK-3β was shown to be independent of Src/EGF receptor transactivation and MAP kinase activation, but similar to other GPCRs (Murga et al., 1998) to occur through phospho-inositol-3-kinase (PI3K) activation via the $G\beta\gamma$-subunits of $G\alpha_{i/o}$-proteins. In the CNS, the Akt/GSK-3β axis plays a prominent role in brain function and has been implicated in neuronal migration, protection against neuronal apoptosis (Brazil et al., 2004) and is believed to be altered in Alzheimer's

disease, neurological disorders (Li et al., 2002; Rickle et al., 2004) and schizophrenia (Emamian et al., 2004). Because the high expression level of the H_3R is restricted to specific areas of the brain during development (Karlstedt et al., 2003), one could speculate that the activation of Akt might be relevant for a H_3R-mediated neuronal migration during development of the CNS exerting its effect through the Akt/GSK-3β pathway, or the MAPK kinase pathway as described above. It has been reported before that simultaneous MAPK and Akt activation are required for cortical neuron migration (Segarra et al., 2006). There is also evidence that the H_3R plays a neuroprotective role in the CNS (Adachi et al., 1993). Moreover, H_3R mRNA is upregulated in certain brain areas after induction of ischemia (Lozada et al., 2005) and kainic acid-induced seizures (Lintunen et al., 2005). Upregulation of the H_3R and the subsequent constitutive signaling to the Akt/GSK-3β pathway could be the mechanism by which the H_3R exerts its endogenous neuroprotective role.

Modulation of intracellular Ca^{2+}

In human neuroblastoma SH-SY5Y cells it was shown that H_3R activation reduced the K^+-induced intracellular calcium mobilization (Figure 4). This signal transduction mechanism was subsequently linked to inhibitory effect of the H_3R on the norepinephrine exocytosis in these cells as well as in cardiac synaptosomes (Silver et al., 2002). In latter studies this effect on K^+-induced calcium mobilization was linked to the H_3R-mediated inhibition of PKA activity, leading to a decreased Ca^{2+} influx through voltage-operated Ca^{2+} channels (Seyedi et al., 2005). No effects on the intracellular Ca^{2+} levels were observed upon administration of H_3R agonists before the K^+-induced calcium release. In contrast, in SK-N-MC cells the heterologous expression of the hH_3R results in a rapid, but transient $G_{i/o}$-protein dependent calcium mobilization from intracellular stores upon the administration of H_3R agonists (Bongers et al., 2006). This observation is analogous to the reported signalling of the related $G_{i/o}$-coupled H_4 receptor, which mobilizes calcium in mast cells and eosinophils (Raible et al., 1994; Hofstra et al., 2003). Further research is needed to study the detailed molecular pathway of the H_3R-mediated calcium mobilization in SK-N-MC cells and determine if similar findings are evident in other cell types that endogenously express H_3Rs.

Inhibition of Na^+/H^+ exchanger activity

The Na^+H^+ exchanger (NHE) is essential for the restoration of intracellular physiological pH by the removal of one intracellular H^+ for one extracellular Na^+ and thereby preventing acidification during ischemia (Karmazyn, 1999). The consequential increase of interneuronal Na^+ forces the reversal of the Na^+- and Cl^--dependent norepinephrine transporter and leads to an increase in carrier-mediated

norepinephrine release. Activation of the H_3R was shown to diminish neuronal NHE activity (Figure 4) and this pathway was proposed as the mechanism by which the H_3R inhibits the excessive release of norepinephrine during protracted myocardial ischemia (Silver et al., 2001). For that reason H_3R agonists were proposed to have therapeutic potential for myocardial ischemia, the negative modulation on norepinephrine release might prevent arrhythmias and sudden cardiac death (Levi and Smith, 2000).

Not much is known about the mechanism by which the H_3R inhibits NHE activity. In general, GPCRs are known to activate NHE through kinases like MAPK (Wang et al., 1997). However, little is know about the signaling mechanisms of GPCRs that attenuate NHE activity (Avkiran and Haworth, 2003), although a direct interaction of $G_{\alpha i}$-proteins has been suggested to be involved in the inhibition of NHE (Siffert et al., 1990; van Willigen et al., 2000).

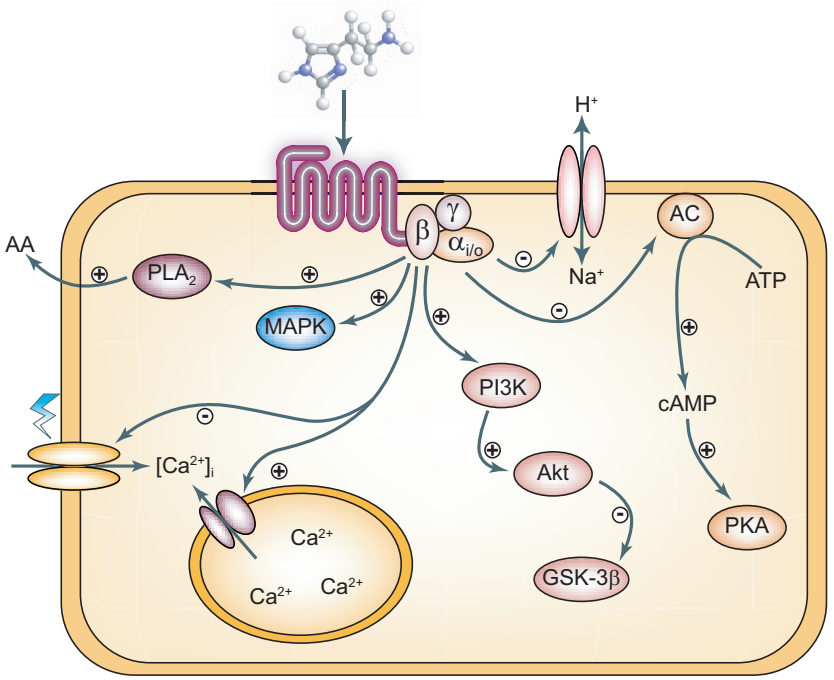

Figure 4. A schematic representation of the H_3R-mediated signal transduction. The H_3R has been shown to modulate several signal transduction pathways including the inhibition adenylyl cyclase (AC), mitogen-activated protein kinase (MAPK), activation of phospholipase A_2 (PLA_2), intracellular calcium mobilization, activation of the Akt/GSK-3β axis and inhibition of the Na^+/H^+ exchanger.

Expression of the H_3R isoforms

The initial cloning of the hH_3R gene demonstrated that the full length receptor is a $hH_3R(445)$ amino acid G-protein coupled receptor that is found almost exclusively in the brain (Lovenberg et al., 1999). Whether the so far described isoforms indeed play an important role will depend on their expression levels and potential differential expression. Cogé et al. (Cogé et al., 2001) showed by Northern blots analysis a high signal for the $hH_3R(445)$ in thalamus, caudate nucleus, putamen and cerebellum, a lower signal in the amygdala and a faint signal for the substantia nigra, hippocampus and cerebral cortex. No signal was observed in the corpus callosum, spinal cord or in peripheral tissue. Further analysis of hH_3R isoforms was done by RT-PCR, for the $hH_3R(445)$ the results was comparable to the Northern blot analysis. The $hH_3R(415)$ and the $hH_3R(365)$ showed high level expression in the thalamus, caudate nucleus and cerebellum, whereas $hH_3R(329)$ and $hH_3R(326)$ were highly expressed in the amygdala, substantia nigra, cerebral cortex and hypothalamus (Cogé et al., 2001). As described by Wellendorph et al. (Wellendorph et al., 2002) the $hH_3R(373/365)$ isoforms are expressed at a higher level than the $hH_3R(445)$ isoform in the stomach and the hypothalamus using a RT-PCR approach (Wellendorph et al., 2002). The differential expression in the hypothalamus is not consistent with the findings by Cogé et al. (Cogé et al., 2001). Clearly more work needs to be done in this area.

Pharmacological characteristics of H_3R isoforms

Pharmacological characterization of different hH_3R isoforms have been described in two publications (Table 1). Of the six isoforms cloned by Cogé et al. (Cogé et al., 2001), three isoforms ($hH_3R(445)$, $hH_3R(431)$, $hH_3R(365)$) were expressed in CHO cells and pharmacologically characterized, with a focus on the $hH_3R(445)$ and $hH_3R(365)$. The $hH_3R(431)$, which lacks 14 amino acids at the C-terminal end of TM2, showed no [^{125}I]Iodoproxyfan radioligand binding. Whereas this deletion does not affect the key-residues in ligand binding for imidazole containing ligands (D^{114} in TM3 and E^{206} in TM5 (Uveges et al., 2002)), it eliminates the structurally important proline residue characteristic for amine receptors (Ballesteros et al., 2001; Visiers et al., 2002). The 14 amino acid deletion is thereby expected to alter the structural organization and likely affects the [^{125}I]Iodoproxyfan binding. A similar pharmacology was observed for the $hH_3R(445)$ and $hH_3R(365)$ in radioligand binding studies. However, H_3R agonists did not generate a functional response on the $hH_3R(365)$ in cAMP and Ca^{2+} assays, nor in a [^{35}S]GTPγS binding assay. In contrast to the findings by Cogé et al., Wellendorph et al. (Wellendorph et al., 2002) have shown that the $hH_3R(365)$ isoform was functional in a R-SAT™ reporter

assay and displayed higher potency for typical H_3R agonists. Higher agonist potencies have also been observed for the rat H_3R isoforms, with similar deletions in the third intracellular loop 3 (Drutel et al., 2001). The truncated isoforms $hH_3R(301)$ (lacking TM6 and 7) and $hH_3R(200)$ (lacking TM5-7) failed to show a biological response, or N^{α}-[^{3}H-methyl]-histamine radioligand binding, not surprising as these isoform lack many residues important for GPCR structure and function (Kristiansen, 2004). However these isoforms can play a role in H_3R signaling as was recently published for the non-functional rat H_3R isoforms lacking TM7, that were shown to act as dominant negatives on the expression of the functional rat H_3R isoforms (Bakker et al., 2006). The $hH_3R(373)$ isoform, which corresponds to $hH_3R(365)$ with eight additional amino acids at the C-terminus, behaves as the $hH_3R(365)$ in the R-SAT™ reporter assay, suggesting that 8 extra amino acids do not dramatically alter the pharmacology of the isoforms. The $hH_3R(409)$, $hH_3R(395)$, $hH_3R(379)$, $hH_3R(329b)$, $hH_3R(293)$ and $hH_3R(290)$ isoforms lack 36 amino acids at the N-terminus, including a N-glycosylation site. Glycosylation at GPCRs have been found to be important in the stabilization dimers (Michineau et al., 2006) and for correct trafficking to the membrane (Rands et al., 1990; Servant et al., 1996; Lanctot et al., 2005), however at this moment is it not known if these 36 amino acids affect hH_3R trafficking or its pharmacology.

Dimerization of H_3Rs

The concept of GPCR dimerization is now well documented in literature (see Pfleger and Eidne, 2005 for a review (Pfleger and Eidne, 2005)), and such direct protein-protein interactions between different GPCRs are suggested to allow a whole vista of possibilities for subtle changes in the pharmacology of these GPCRs from their monomeric, homo-dimeric or -oligomeric entities, which were previously attributed to the existence of additional receptor subtypes. In view of the recent discovery of H_3R isoforms, which are often co-expressed, the occurrence of H_3R isoform dimerization might add another level of complexity to the H_3R pharmacology.

The first evidence for H_3R isoform dimerization comes from the use of an antibody directed against the rat $H_{3C}R$ isoform (48 amino acid deletion in I3) using both native as well as heterologously expressed rat H_3Rs (Shenton et al., 2005). Subsequent *time-resolved* Fluorescent Resonance Energy Transfer (*tr*-FRET) experiments using heterologously expressed epitope tagged rat $H_{3A}Rs$ have shown the presence of oligomeric rat $H_{3A}Rs$ at the cell surface (Bakker et al., 2006). As many of the functional H_3R isoforms differ in the length of their third intracellular loop, one could envision that this might influence the capabilities of the isoforms to form domain-swap H_3R (homo- or hetero-) dimers. In addition, sequences within

the third intracellular loop may serve a scaffolding function. The potential protein-protein interaction involving various functional H_3R isoforms remains unclear.
In addition to the functional H_3R isoforms, several (presumed) non-functional H_3R isoforms have been detected. These non-functional isoforms consist either of a truncated receptor comprising only the proximal part of a full-length isoform, the amino terminal domain until the second transmembrane domain, or of a C-terminal truncated isoform that in comparison to a full length isoform lacks transmembrane 7 and have been named 6TM-H_3R isoforms (Bakker et al., 2006). For these truncated isoforms neither the binding of known H_3R radioligands nor any functional responses have been observed. The roles of these non-functional H_3R isoforms are poorly understood. Intriguingly, mRNAs coding for the truncated and assumed non-functional rat H_3R isoforms are expressed in the brain to a similar extent as the functional rat H_3R isoforms (Morisset et al., 2001; Bakker et al., 2006), suggesting that these truncated proteins may have yet unidentified functions. Recently, we reported on the identification of three rat 6TM-H_3R isoforms which are capable to specifically negatively influence the cell surface expression of the full length functional H_3R isoforms, and that mRNA expression of these 6TM-H_3R isoforms are modulated upon treatment with the convulsant pentylenetetrazole. These observations corroborate the potential functional importance of the otherwise non-functional truncated H_3R isoforms (Bakker et al., 2006). The 6TM-H_3R isoforms appear to affect the cell surface expression of the functional isoforms through retention of these functional isoforms within the cell. Retention occurs most likely within the endoplasmatic reticulum, probably through the formation of heterodimeric H_3Rs consisting of a mixture of functional and non-functional isoforms that lack domains that are required for appropriate interactions with accessory proteins that mediate the cell-surface targeting of the receptor complex. In evidence for this, the 6TM-rH_3R isoforms lack a F(X)6LL motif that is reported to be important for interaction with a specific ER-membrane-associated protein that regulates transport of GPCRs (Bermak et al., 2001), and possess an RXR ER retention signal instead (Bakker et al., 2006).

Concluding remarks

The cloning of the hH_3R has led to the discovery of several signal transduction pathways that are modulated by the hH_3R. Some of these signaling pathways can be linked to relevant pathophysiologies. The hH_3R-mediated inhibition of the NHE leads to a subsequent lowering in the exocytosis of norepinephrine and thereby providing an explanation for the protective role of hH_3R agonists during myocardial ischemia. The cloning of the receptor gene resulted in the elucidation of the genomic organization of the hH_3R and the discovery of many hH_3R isoforms. To

date there is limited knowledge on the regulation of the expression of the H_3R isoforms or regulation of the alternative splicing of H_3R mRNA. The pharmacology and signal transduction of these isoforms is also still largely unknown and needs further investigation. Moreover, G-protein independent signaling of the H_3R, e.g. by G-protein coupled receptor kinases or β-arrestin, has not been studied and might provide additional insight in molecular aspects of the H_3R isoforms. Surprisingly, even non-signaling isoforms have been shown to have a physiological role by influencing the expression of several functional H_3R isoforms. Moreover, little is known about the hH_3R in relation the homo/heterodimerization and the existence of the hH_3R isoforms. GPCRs are shown to form non-covalent dimers through hydrophobic interactions between helices or coiled-coil structures; deletion of certain sequences in the hH_3R protein is likely to have an effect on the formation of dimers. In view of the suggested H_3R heterogeneity in both functional and radioligand binding studies, the occurrence of H_3R isoforms and (potential) dimerization might provide some of the molecular explanations.
However, how intricate the molecular details of the hH_3R might have become, the recent progress to clinical phase studies shows the therapeutic potential of hH_3R inverse agonists and substantiates that the H_3R is a promising drug target.

Aim of this thesis

The aim of this thesis is to get a more detailed understanding on the molecular pharmacology of the H_3R. Cloning of the H_3R allowed for the recombinant expression of H_3Rs and its isoforms in cell lines facilitating the study of H_3R-mediated signal transduction and pharmacology.

The aims of this thesis can be summarized as follows:

- Evaluate constitutive activity of the H_3R
- Investigate novel signal transduction pathways activated by the H_3R
- Pharmacological characterization of the two predominantly expressed human H_3R isoforms, the $hH_3R(445)$ and $hH_3R(365)$
- Study the effects of the expression of specific $G\alpha_{i/o}$-proteins on the pharmacology of H_3R isoforms having deletions in the third intracellular loop.

Chapter 3 describes the pharmacological evaluation of the H_3R recombinantly expressed in SK-N-MC cells and provides proof for the constitutive activity of the H_3R, which led to the reclassification of heretofore known antagonists to inverse agonists and the discovery of a neutral H_3R antagonist. Chapter 4 describes the pharmacological characterization of two highly expressed human H_3R isoforms, the $H_3R(445)$ and $H_3R(365)$, and the discovery that the $H_3R(365)$ shows are marked increase in constitutively active compared to the $H_3R(445)$ and that this feature affects their mutual pharmacology. Chapter 5 describes the investigation of various $G\alpha_{i/o}$-proteins on the pharmacology of the H_3R isoforms. Chapter 6 and 7 describe the discovery of two novel H_3R-mediated signal transduction pathways, the activation of Akt/GSK-3β axis and the release of $[Ca^{2+}]_i$, respectively.

Chapter 3

Constitutive activity of histamine H_3 receptors stably expressed in SK-N-MC Cells: display of agonism and inverse agonism by H_3 antagonists

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Abstract

Agonist-independent activity of G-protein-coupled receptor, also referred to as constitutive activity, is a well-documented phenomenon and has been reported recently for both the histamine H_1 and H_2 receptors. Using SK-N-MC cell lines stably expressing the human and rat H_3 receptors at physiological receptor densities (500-600 fmol/mg of protein), we show that both the rat and human H_3 receptors show a high degree of constitutive activity. The forskolin-mediated cAMP production in SK-N-MC cells is inhibited strongly upon expression of the G_i-coupled H_3 receptor. The cAMP production can be further inhibited upon agonist stimulation of the H_3 receptor and can be enhanced by a variety of H_3 antagonists acting as inverse agonists at the H_3 receptor. Thioperamide, clobenpropit, and iodophenpropit raise the cAMP levels in SK-N-MC cells with potencies that match their receptor binding affinities. Surprisingly, impentamine and burimamide act as effective H_3 agonists. Modification of the amine group of impentamine dramatically affected the pharmacological activity of the ligand. Receptor affinity was reduced slightly for most impentamine analogs, but the functional activity of the ligands varied from agonist to neutral antagonist and inverse agonist, indicating that subtle changes in the chemical structures of impentamine analogs have major impact on the (de)activation steps of the H_3 receptor. In conclusion, upon stable expression of the rat and human H_3 receptor in SK-N-MC cells constitutive receptor activity is detected. In this experimental system, H_3 receptors ligands, previously identified as H_3 antagonists, cover the whole spectrum of pharmacological activities, ranging from full inverse agonists to agonists.

Introduction

The histamine H_3 receptor was discovered in 1983 by Arrang and coworkers as a presynaptic autoreceptor regulating the release of histamine from histaminergic neurons (Arrang et al., 1983). Since then, the H_3 receptor has been shown to act as heteroreceptor as well, inhibiting the release of important neurotransmitters, e.g., acetylcholine, glutamate, noradrenaline, and serotonin (Leurs et al., 1998). With the availability of a variety of selective and potent H_3 agonists and antagonists (Leurs et al., 1995; Stark et al., 1996), it has become clear that the H_3 receptor is involved in the regulation of several important physiological processes. Consequently, the H_3 receptor is regarded as an interesting target for the modulation of a variety of functions such as cognitive processes, epilepsy, food intake, and sleep-wakefulness (Leurs et al., 1998).

Despite the interest in H_3 receptor ligands for therapeutic application, the actual therapeutic development has for a long time been hampered by the lack of

information on the molecular target. Whereas the cloning of the H_1 and H_2 receptor genes was reported in the early 90s (Gantz et al., 1991b; Yamashita et al., 1991), it was 1999 before the gene of the human H_3 receptor was cloned finally by Lovenberg et al. (1999) after the identification of a partial sequence of an orphan G-protein-coupled receptor (GPCR) in the Incyte expressed sequence tags database. The H_3 receptor was shown finally to be a GPCR with only limited homology (<30%) with the H_1 and H_2 receptor genes (Lovenberg et al., 1999).
Classical models of GPCRs require agonist occupation of receptors to activate signal transduction pathways. Yet, it is now well-documented that GPCRs can be spontaneously active, and this agonist-independent receptor activity is often referred to as constitutive receptor activity (Costa et al., 1992; Lefkowitz et al., 1993; Milligan et al., 1995). Inverse agonists reduce the constitutive GPCR activity, whereas neutral antagonists do not affect the basal GPCR activity but prevent the action of both agonists and inverse agonists. Constitutive activity has been shown recently for both the histamine H_1 and H_2 receptors (Smit et al., 1996; Bakker et al., 2000), and we reported that the therapeutically important H_1 and H_2 antagonists, in fact, act as inverse agonists. In the present study, we describe that the human and rat histamine H_3 receptors stably expressed in SK-N-MC cells (Lovenberg et al., 1999; Lovenberg et al., 2000) show a high level of constitutive activity, resulting in the identification of several standard H_3 antagonists (thioperamide and clobenpropit) as inverse agonists in this cell system. Moreover, burimamide and impentamine, previously identified as H_3 antagonists (Arrang et al., 1983; Vollinga et al., 1995b; Vollinga et al., 1995a), behave as H_3 agonists at the recombinant H_3 receptors. The agonistic effects of impentamine could also be demonstrated on the hypothalamic histamine release in the rat brain using in vivo microdialysis. Moreover, in a series of impentamine analogs we were able to manipulate the intrinsic activity. VUF4904, an impentamine analog with an isopropyl group at the amino group of the side chain, bound with a relatively high affinity (12 nM) and acted as a neutral antagonist in the transfected SK-N-MC cells. These data indicate that ligands, previously identified as H_3 antagonists, can cover the whole spectrum of pharmacological activities, ranging from full inverse agonism to agonism, at the recombinant H_3 receptors heterologously expressed in SK-N-MC cells.

Results

The generation of the SK-N-MC cells stably expressing either the rat or human histamine H_3 receptor was described previously (Lovenberg et al., 1999; Lovenberg et al., 2000). In the present study, we used SK-N-MC cell lines, expressing 516 ± 23 fmol/mg of protein (n = 3) of the human histamine H_3 receptor

or 627 ± 87 fmol/mg of protein (n = 3) of the rat histamine H_3 receptor, as assessed by [^{3}H]*N*$^\alpha$-methylhistamine binding.

As described previously, the H_3 agonists (*R*)-α-methylhistamine (pEC_{50} = 9.26 ± 0.08) and imetit (pEC_{50} = 9.28 ± 0.04) potently inhibited the 10 µM forskolin-stimulated production of cAMP in human H_3 receptor expressing cells (Figure 1A; Table 1). In contrast, (*R*)-α-methylhistamine had no effect in the parental SK-N-MC cell line (Figure 1A). As expected for a G_i-coupled receptor, the 1 µM (*R*)-α-methylhistamine effects at the human H_3 receptor (reduction to 11 ± 3% of the forskolin-induced cAMP levels) were abolished completely by an overnight pretreatment with 100 ng/ml pertussis toxin (99 ± 10% of forskolin-induced cAMP level, not shown).

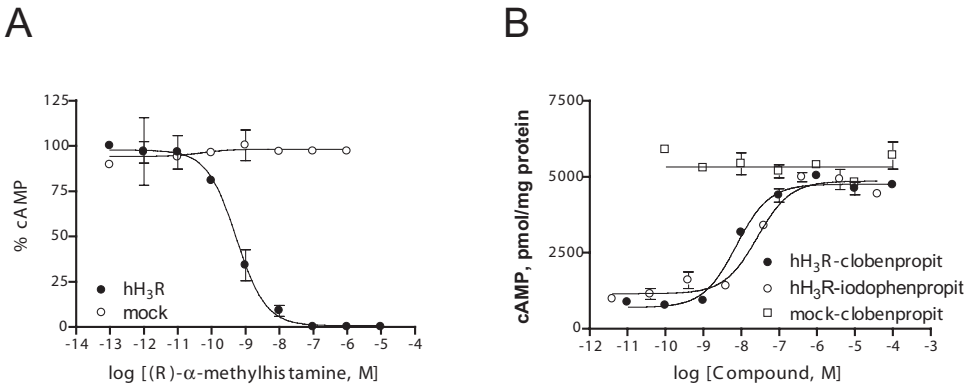


Figure 1. Modulation of 10 µM forskolin induced cAMP production in SK-N-MC cells, expressing the human H_3 receptor. A) effects of the H_3 agonist (*R*)-α-methylhistamine on the forskolin response in SK-N-MC cells or SK-N-MC cells expressing the human H_3 receptor. B) effects of the H3 inverse agonists clobenpropit and iodophenpropit on the forskolin response in SK-N-MC cells expressing the human H_3 receptor. For comparison, the effects of clobenpropit on nontransfected SK-N-MC cells is shown. Cells were incubated for 10 min with the indicated ligands. After termination of the incubation the cAMP levels were determined by a competitive binding assay.

Table 1. H_3 receptor affinity (pK_i) was determined by [^{3}H]-N$^{\alpha}$-methylhistamine binding to membranes of SK-N-MC cells expressing the human H_3 receptor. For agonists the pEC_{50} values were determined by the inhibition of the forskolin-stimulated (10 µM) cAMP production, whereas the inverse agonistic activity was determined by the increase of the forskolin response in SK-N-MC cells, expressing the human H_3 receptor. All data shown are the mean ± S.E.M. of at least three experiments. *indicates a significant difference compared with (*R*)-α-methylhistamine. **indicates a significant difference compared with iodophenpropit.

Ligand	pK_i	pEC_{50}	α
(*R*)-α-Methylhistamine	8.4 ± 0.1	9.3 ± 0.1	1.0 ± 0.05
Imetit	8.8 ± 0.1	9.3 ± 0.1	1.0 ± 0.02
VUF8328	8.5 ± 0.0	8.9 ± 0.1	0.9 ± 0.03
Impentamine	8.3 ± 0.1	8.6 ± 0.2	0.9 ± 0.08
Burimamide	7.1 ± 0.1	6.7 ± 0.1	0.8 ± 0.09*
Iodophenpropit	8.2 ± 0.1	7.6 ± 0.1	-1.0 ± 0.07
Thioperamide	7.2 ± 0.0	6.7 ± 0.2	-0.7 ± 0.1**
Clobenpropit	8.4 ± 0.0	8.4 ± 0.2	-0.9 ± 0.1

Interestingly, we noticed an important difference between the forskolin-induced cAMP levels of the parental SK-N-MC cell line and the H_3 receptor expressing cells (Figure 1B). As this could be an indication of constitutive H_3 receptor activation, we tested a variety of previously identified H_3 antagonists on the SK-N-MC cell line expressing the human H_3 receptor. Standard H_3 antagonists as thioperamide, clobenpropit, and iodophenpropit concentration-dependently increased the forskolin-induced cAMP levels in the transfected SK-N-MC cells (Figure 1B; Table 1), whereas clobenpropit had no effect on the forskolin response in the parental cell line (Figure 1B). In our experiments, iodophenpropit and clobenpropit acted as full inverse agonists (α = -1.0-0.9), whereas thioperamide acted as a partial inverse agonist (Table 1). The obtained pEC_{50} values for the various inverse agonists correspond well with the respective affinities, as obtained in [^{3}H]N$^{\alpha}$-methylhistamine competition experiments (Table 1).

In search of neutral antagonists, we tested a variety of other H_3 antagonists. Surprisingly, the presumed H_3 antagonists burimamide, impentamine, and the imetit homolog VUF 8328 (van der Goot et al., 1992) all acted as potent H_3 agonists at the human H_3 receptor with intrinsic activities between 0.8 and 0.9 (Figure 2; Table 1). Comparing the pK_i values of various agonists with their pEC_{50} values revealed that in general the potencies of the agonists nicely parallel their affinities (Table 1). Only for the full agonists (*R*)-α-methylhistamine and perhaps imetit may some sort of receptor reserve be noticed (Table 1).

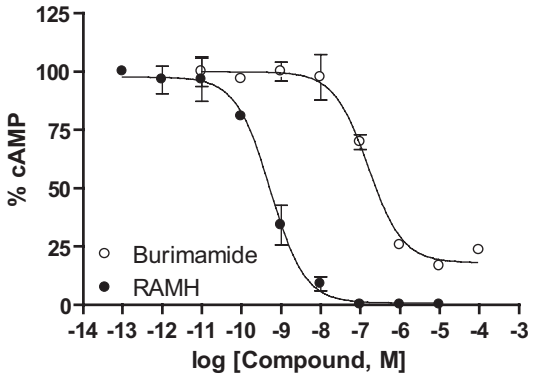
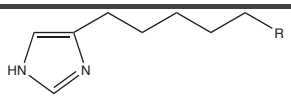
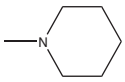
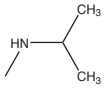
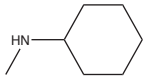
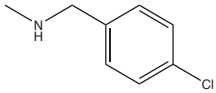


Figure 2. Agonistic activity of (*R*)-α-methylhistamine (RAMH) and burimamide in SK-N-MC cells expressing the human H_3 receptor. SK-N-MC cells expressing the human H_3 receptor were stimulated with 10 μM forskolin in the presence of increasing concentrations of the H_3 ligands for 10 min. After termination of the incubation the cAMP levels were determined by a competitive binding assay.

Based on the identification of agonism of impentamine, we studied several impentamine analogs to identify a neutral H_3 receptor antagonist. In this series of H_3 ligands, the amine function of impentamine was substituted or incorporated in a piperidine ring. Modification of the amine group results in a series of compounds with a wide spectrum of pharmacological activity, including the neutral antagonist VUF4904 (Figure 3; Table 2).

Table 2. Affinities and functional activities of several impentamine analogs at the human H_3 receptor. H_3 receptor affinity (pK_i) was determined by [^{3}H]-*N*$^\alpha$-methylhistamine binding to membranes of SK-N-MC cells expressing the human H_3 receptor. For agonists the pEC_{50} values were determined by the inhibition of the forskolin-stimulated (10 µM) cAMP production, whereas the inverse agonistic activity was determined by the increase of the forskolin response in SK-N-MC cells, expressing the human H_3 receptor. All data shown are the mean ± S.E.M. of at least three experiments.

Ligand		**pK_i**	**pEC_{50}**	**α**
Impentamine		8.3 ± 0.1	8.6 ± 0.2	0.9 ± 0.08
VUF5300		8.0 ± 0.1	8.7 ± 0.2	1.0 ± 0.02
VUF5207		7.8 ± 0.2	7.9 ± 0.1	0.7 ± 0.05[a]
VUF4904		7.9 ± 0.1		-0.1 ± 0.1[a]
VUF4903		8.0 ± 0.0	8.1 ± 0.2	-0.6 ± 0.1[b]
VUF5202		8.6 ± 0.1	8.7 ± 0.1	-0.9 ± 0.1

[a]Indicates a significant difference compared with (R)-α-methylhistamine.
[b]Indicates a significant difference compared with iodophenpropit.

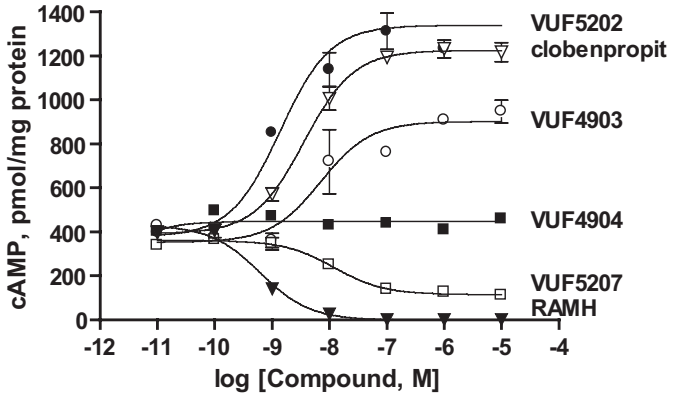


Figure 3. Modulation of 10 μM forskolin induced cAMP production in SK-N-MC cells expressing the human H_3 receptor by a variety of impentamine analogs. SK-N-MC cells expressing the human H_3 receptor were stimulated with 10 μM forskolin in the presence of increasing concentrations of the H_3 ligands for 10 min. For comparison the effects of clobenpropit and (*R*)-α-methylhistamine are shown as well. After termination of the incubation the cAMP levels were determined by a competitive binding assay.

Constitutive activity is not restricted to the human H_3 receptor. Whereas the rat and human H_3 receptor were expressed at similar levels (627 fmol/mg of protein versus 516 fmol/mg of protein), the forskolin-induced cAMP levels were always lower in the SK-N-MC cells expressing the human H_3 receptor (Figure 4). These data indicate that in our experimental model the level of constitutive activity of the human H_3 receptor is more pronounced than that of the rat H_3 receptor. At the recombinant rat receptor, the H_3 ligands burimamide and impentamine also behave as H_3 agonists. In contrast to the human H_3 receptor, both ligands behave as full agonists at the rat H_3 receptor (Table 3). The constitutive activity, displayed by the rat H_3 receptor, can also be inhibited by compounds such as clobenpropit (Figure 4; Table 3). Especially for the inverse agonists thioperamide and iodophenpropit, we confirmed the reported species differences (Lovenberg et al., 1999; Lovenberg et al., 2000) with respect to their potencies in both receptor binding and functional assays (Table 3).

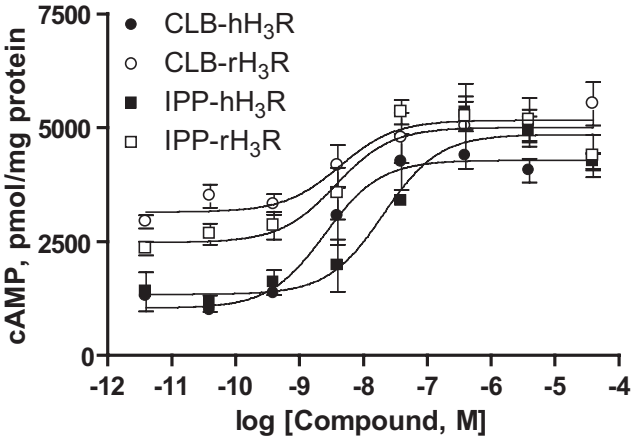


Figure 4. Modulation of 10 µM forskolin induced cAMP production in SK-N-MC cells expressing the human or rat H_3 receptor, by the inverse agonists clobenpropit or iodophenpropit. SK-N-MC cells were stimulated with 10 µM forskolin in the presence of increasing concentrations of H_3 ligand for 10 min. After termination of the incubation, the cAMP levels were determined by a competitive binding assay.

Table 3. Affinities and functional activities of several H_3 receptor ligands at the rat and human H3 receptor. H_3 receptor affinity (pKi) was determined by [^{3}H]-N$^\alpha$-methylhistamine binding to membranes of SK-N-MC cells expressing the human or rat H_3 receptor. For agonists the pEC_{50} values were determined by the inhibition of the forskolin-stimulated (10 µM) cAMP production, whereas the inverse agonistic activity was determined by the increase of the forskolin response in SK-N-MC cells, expressing the H_3 receptor. All data shown are the mean ± S.E.M. of at least three experiments.

Ligand	Human H_3			Rat H_3		
	pK_i	pEC_{50}	α	pK_i	pEC_{50}	α
(*R*)-α-Methylhistamine	8.4±0.1	9.3±0.1	1.0±0.05	8.0±0.1	9.2±0.2	1.0±0.10
Impentamine	8.3±0.1	8.6±0.2	0.9±0.08	8.3±0.3	9.0±0.1	1.1±0.1
Burimamide	7.1±0.1	6.7±0.1	0.8±0.09[a]	7.3±0.1	7.2±0.0	1.1±0.02
Iodophenpropit	8.2±0.1	7.6±0.1	-1.0±0.07	8.8±0.1	8.0±0.2	-1.0±0.05
Clobenpropit	8.4±0.0	8.4±0.2	-0.9±0.1	9.0±0.1	8.5±0.1	-1.0±0.1
Thioperamide	7.2±0.0	6.7±0.2	-0.7±0.1[b]	8.2±0.0	8.1±0.4	-1.0±0.07

To investigate the predictive value of the data obtained with the recombinant receptors, impentamine and clobenpropit were tested in vivo. Previously, we showed by microdialysis the H_3 receptor-mediated effect on in vivo histamine release in the rat hypothalamus (Jansen et al., 1998). Using the same experimental set-up, we first evaluated the effects of impentamine. The mean values ± S.E.M. of the basal histamine release in the experiments in Figure 5 were 0.078 ± 0.008 (n = 4) pmol/20 min. This value remained constant throughout the experimental period

of 5 h under anesthesia (data not shown). After infusion of impentamine the histamine levels in the hypothalamus rapidly decreased to approximately 40% of the basal levels. Concomitant infusion of clobenpropit reversed the effect of impentamine and even caused an increase (±40%) in the histamine release above basal levels (Figure 5).

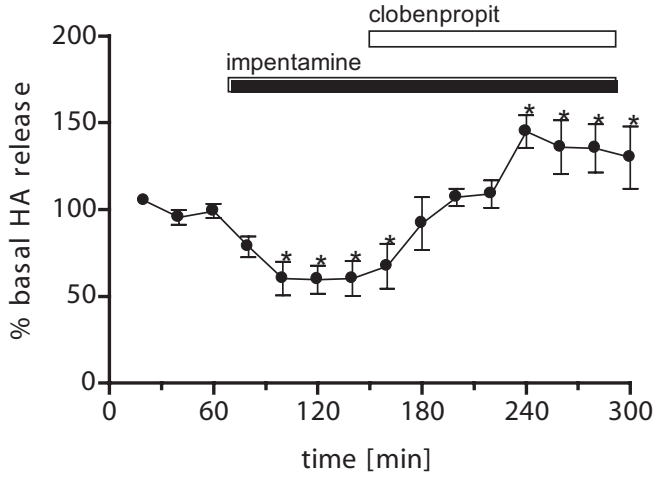


Figure 5. Effect of impentamine and clobenpropit on the *in vivo* histamine release in the rat hypothalamus as measured by microdialysis. Drugs (10 μM) were infused in the hypothalamus via the microdialysis probe. Fractions were collected every 20 min and the amount of histamine was determined by HPLC, as described under Experimental Procedures. *, a significant ($P < 0.05$) difference compared with basal levels of histamine release.

The recent cloning of the rat and human H_3 receptor cDNAs by Lovenberg et al. (1999, 2000) has had a great impact in the field of histamine research. The new information has been instrumental in identifying H_3 receptor isoforms <Drutel, 2001 #101> and the H_4 receptor (Nakamura et al., 2000; Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001) and has also been essential in deriving important information about the signaling properties of the H_3 receptor. Whereas for many years the actual signaling pathways for the H_3 receptor had been unknown, the use of cell lines expressing the H_3 receptor has led to the identification of at least three signal transduction pathways for the H_3 receptor: a G_i-mediated inhibition of adenylate cyclase (Lovenberg et al., 1999; Drutel et al., 2001), the activation of the MAP kinase pathway (Drutel et al., 2001), and the stimulation of Na^+/H^+ exchange (Silver et al., 2001).
Using SK-N-MC cell lines, stably expressing either the human and rat H_3 receptors at physiological receptor densities (500-600 fmol/mg of protein) (Yanai et al., 1994;

Brown et al., 1996), we now show that both the rat and human H_3 receptor display a high degree of constitutive activity. The forskolin-mediated cAMP production in SK-N-MC cells is inhibited strongly upon expression of the G_i-coupled H_3 receptor. The cAMP production can be further inhibited upon agonist stimulation of the H_3 receptor and can be enhanced by a variety of H_3 antagonists acting as inverse agonists at the H_3 receptor. Thioperamide, clobenpropit, and iodophenpropit raise the cAMP levels in SK-N-MC cells expressing either the human or rat H_3 receptor with potencies that match their receptor binding affinities. As reported previously (Lovenberg et al., 1999; Lovenberg et al., 2000), an important species difference is noticed for thioperamide in both the binding and cAMP assay.

Burimamide was one of the key compounds used by Arrang et al. (1983) to demonstrate pharmacologically the existence of the H_3 receptor in rat cerebral cortex slices. Remarkably, at recombinant H_3 receptors, the presumed H_3 antagonist burimamide acts as a H_3 agonist. Whereas at the human H_3 receptor burimamide acts as a partial agonist (α = 0.8), full agonism is observed at the rat receptor. It is interesting to note that via the use of heterologous expression systems, burimamide is reclassified for the second time. Previously, we showed that at the human H_2 receptor burimamide acts as a weak partial agonist (Alewijnse et al., 1998). Because burimamide was developed originally as an H_2 antagonist using histamine as a starting point (Black et al., 1972a), the discovery of residual agonistic activity at histamine H_2 and H_3 receptors is perhaps not too surprising.

The use of transfected cell lines also suggests a reclassification for impentamine, the histamine homolog previously suggested to differentiate between H_3 receptors in the guinea pig intestine and rat or guinea pig brain (Leurs et al., 1996; Harper et al., 1999). Impentamine is a potent H_3 antagonist in the guinea pig intestine (pA_2 = 8.4), but a partial agonist in the rat brain (pD_2 = 8.2, α= 0.6) (Leurs et al., 1996). Moreover, radioligand binding studies at the H_3 receptor in the guinea pig brain and intestine indicated that impentamine can discriminate between the receptors in the two preparations (Harper et al., 1999). At both the recombinant rat and human H_3 receptors, impentamine behaves as an effective agonist. Moreover, in vivo microdialysis shows that impentamine also acts as an H_3 agonist in the rat hypothalamus, inhibiting the basal release of histamine. Previously we showed that potent H_3 agonists, like immepip (Jansen et al., 1998), inhibit the hypothalamic histamine release to approximately the same extent as observed in this study for impentamine.

Although constitutive GPCR activity is now a widely accepted pharmacological concept, effects due to the presence of the natural agonist cannot be ignored completely. The identification of neutral antagonists has resolved this issue for the histamine H_2 receptor and led to the recognition that the therapeutically important H_2 antagonists are in fact inverse agonists (Smit et al., 1996). To identify a neutral

H_3 antagonist we tested a variety of impentamine analogs at the human H_3 receptor. The amine function of impentamine probably interacts with the aspartate residue Asp^{114} in transmembrane domain 3, which is highly conserved in the family of biogenic amines (de Esch et al., 2000). We hypothesized that modification of the amine function potentially could affect the agonistic properties of impentamine. Indeed, modification of the amine group dramatically affected the pharmacological activity of the ligand. Receptor affinity was reduced slightly for most analogs, unless a p-chloro-benzyl group was used (VUF5205, pKi = 8.63). Remarkably, introduction of small alkyl groups resulted in reduced agonistic activity (di-methyl substitution, VUF5207, α = 0.7) or neutral antagonism (isopropyl substitution, VUF4904). Substitution of the amine group with a cyclohexyl ring or a p-chlorobenzyl group resulted in (partial) inverse agonists. Our data show that only subtle changes at the amine function alter the pharmacological activity of the ligands. At present, we do not have an explanation for this phenomenon, but this series of ligands may be of great help to understand the mechanism of receptor (in)activation. Detailed studies with receptor mutants, the development of similar, rigid analogs and the generation of a three-dimensional computer model to rationalize receptor-ligand interaction may also be useful in this respect.

In conclusion, in this study we show that both the rat and human H_3 receptors show a considerable level of constitutive activity when expressed at physiological expression levels in SK-N-MC cells. This observation has important consequences for the classification of H_3 receptor ligands, which can now be classified as inverse agonists, neutral antagonists, and agonists.

Constitutive activity of the rat H_3 receptor was also reported very recently by Morisset et al. (2000). Interestingly, the constitutive activity of the rat H_3 receptor was suggested to regulate brain histamine release in both rat and mouse (Morisset et al., 2000). The H_3 receptor is, therefore, one of the few GPCRs for which it is known that they modulate important physiological processes by means of its constitutive activity. In light of the foreseen therapeutic application of H_3 antagonists (Leurs et al., 1998), it remains to be established whether inverse agonists or neutral antagonists will be favored for clinical application.

Materials and Methods

Materials. (*R*)-α-Methylhistamine dihydrobromide was obtained from Sigma Research Biochemicals Inc. (Zwijndrecht, The Netherlands). Burimamide was a kind gift of GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK). All other H_3 ligands were taken from laboratory stock or (re-)synthesized at the Vrije Universiteit Amsterdam (details will be published elsewhere). Forskolin, 3-isobutyl-1-methylxanthine, cyclic 3',5'-adenosine monophosphate (cAMP), pertussis toxin, and bovine serum albumin were obtained from

Sigma. Dulbeccos's modified Eagle's medium, trypsin-EDTA, penicillin, nonessential amino acids, L-glutamine, streptomycin, and sodium-pyruvate were from Invitrogen (Breda, The Netherlands). Eagle's minimal essential medium was from BioWhittaker (Verviers, Belgium), and fetal calf serum was from Integro (Zaandam, The Netherlands). Culture dishes and 24-well plates were from Costar (Haarlemermeer, The Netherlands). G418 was obtained from Calbiochem (Amsterdam, The Netherlands). [^{3}H]cyclic 3',5'-adenosine monophosphate ([^{3}H]cAMP), 40 Ci/mmol, was from Amersham (s'Hertogenbosch, The Netherlands); [^{3}H]N$^\alpha$-methylhistamine, 85 Ci/mmol, was from PerkinElmer Life Sciences (Zaventem, Belgium).

Cell Culture. SK-N-MC cells, a human neuroblastoma cell line stably expressing the human histamine H_3 receptor (the 445-amino acid isoform) or the rat histamine H_3A receptor (Lovenberg et al., 1999, 2000), were grown in 10-cm2 dishes at 37°C in a humidified atmosphere with 5% CO_2 in Eagle's minimal essential medium, supplemented with 10% v/v fetal calf serum, 50 IU/ml penicillin, nonessential amino acids, 2 mM L-glutamine, 50 µg/ml streptomycin, and 50 µg/ml sodium-pyruvate in presence of 600 µg/ml G418. Cells were detached from the dishes with 0.05% trypsin-EDTA.

[^{3}H]N$^\alpha$-Methylhistamine Binding. Confluent 10-cm dishes of SK-N-MC cells stably expressing the rat or human histamine H_3 receptor were harvested using a cell scraper and centrifuged (3 min, 500g), and the pellets were stored at -20°C until the day of the experiment. Before use the pellets were dissolved in distilled water and homogenized for 2 s by sonication (40 Watt, Labsonic 1510). The cell homogenates (30-100 µg) were incubated for 40 min at 25°C with 1 nM [^{3}H]N$^\alpha$-methylhistamine (85.0 Ci/mmol) in 50 mM sodium phosphate buffer, pH 7.4, with or without competing ligands. The reaction was terminated by rapid dilution with 3 ml of ice-cold buffer, pH 7.4, and filtration over 0.3% polyethylenimine-pretreated Whatmann GF/C filters with two subsequent washes with 3 ml of buffer. Retained radioactivity was determined by liquid scintillation counting. Nonspecific binding was defined with 100 µM thioperamide as competing ligand.
Protein concentrations were determined spectrophotometrically (Packard Argus 400 Microplate Reader) using the Bradford reagent (Bradford, 1976), with bovine serum albumin as a standard.

Measurement of cAMP. SK-N-MC cells stably expressing either the rat or human H_3 receptor were grown overnight in 24-well plates (4.5 × 106 cells/plate), washed once with Dulbeccos's modified Eagle's medium/HEPES (25 mM, pH 7.4 at 37°C), and preincubated in the same medium for 30 min at 37°C. Thereafter the cells were incubated for exactly 10 min with fresh medium supplemented with 0.3 mM 3-isobutyl-1-methylxanthine, 10 µM forskolin, and the respective ligands. To stop the incubation the medium was discarded, 200 µl of ice-cold HCl (0.1 M) was added, and the samples were homogenized for 2 s (40 Watt, Labsonic 1510) and frozen at -20°C.
The intracellular cAMP concentration was determined in a competitive binding assay in which the formed cAMP competes with [^{3}H]cAMP for binding to protein kinase A (Nordstedt and Fredholm, 1990). To this end, plates were thawed quickly and neutralized with 1 M NaOH. To 25 to 200 µl cell homogenate, 50 µl of [^{3}H]cAMP, 200 µl of protein kinase A

suspension, and cAMP assay buffer (Nordstedt and Fredholm, 1990) were added to reach a total volume of 450 µl. After 2.5 h at 4°C, the reaction was terminated by rapid dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4 at 4°C and filtration over Whatmann GF/B filters with two subsequent 3-ml washes. Retained radioactivity was determined by liquid scintillation counting. From a cAMP standard curve (0, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 pmol/100 µl) the amount of cAMP in each sample was calculated using the nonlinear regression-fitting program AssayZap.

In Vivo Microdialysis. Male Wistar rats weighing about 250 g were anesthetized with urethane (1.2 g/kg, i.p.) and placed in a stereotaxic apparatus. A dialysis probe (CMA/10; membrane length, 2 mm; CMA/Microdialysis AB, Stockholm, Sweden) was inserted into the anterior hypothalamic area with coordinates of AP, 1.5; L, 0.5; and V, 9.2 mm relative to the bregma, according to the atlas of Paxinos and Watson (1986). The anterior hypothalamic area was perfused with artificial cerebrospinal fluid containing 140 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose, pH 7.4, through a dialysis probe at 1 µl/min using a microinfusion pump (CMA100, CMA/Microdialysis AB). Two hours after the insertion of a probe, samples were collected every 20 min with a minifraction collector (CMA140, CMA/Microdialysis AB) and frozen immediately at -40°C until analysis. Impentamine and clobenpropit were added to cerebrospinal fluid at the concentration of 10 µM and administered through the dialysis membrane. After the experiment, the brains were removed for histological verification of sites of infusion.
The concentration of histamine in the perfusate was assayed by HPLC (Yamatodani et al., 1985; Mochizuki et al., 1991) The recovery of histamine through the microdialysis membrane is about 40% (Mochizuki et al., 1991).
In each microdialysis experiment, the average of the first three fractions was defined as basal release, and the subsequent fractions were expressed as a percentage of this. The statistical differences between groups were analyzed initially using one-way analysis or variance for repeated measurements. If significant effects versus the basal release were found, data were further analyzed by post hoc Newman-Keuls test.

Data Analysis. For the binding studies pIC_{50} (negative logarithm of the ligand concentration that displaces the radioligand half-maximally) and pKd values (negative logarithm of the equilibrium dissociation constant of the radioligand, i.e., the concentration, that occupies 50% of the available receptors at equilibrium) were calculated using nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA) and converted to pK_i values (negative logarithm of the equilibrium dissociation constant for binding of the unlabeled drug) using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). From the cAMP data pEC_{50} (negative logarithm of the ligand concentration, that activates the receptor half-maximally) and pIC_{50} values (negative logarithm of the ligand concentration, that inhibits the receptor half-maximally) were obtained by fitting these data to a sigmoidal relationship using GraphPad Prism. The intrinsic activities were calculated in comparison with the effects of the full agonist (*R*)-α-methylhistamine (1 µM) or the full inverse agonist iodophenpropit (10 µM).

All data are presented as mean ± S.E.M.; statistical comparisons were performed using the Student's t test.

Chapter 4

A 80 amino acid deletion in the third intracellular loop of a naturally occurring human histamine H_3 isoform confers pharmacological differences and constitutive activity

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Abstract

In this report we pharmacologically characterized two naturally occurring human histamine H_3 receptor (hH_3R) isoforms, the $hH_3R(445)$ and $hH_3R(365)$. These abundantly expressed splice variants differ by a deletion of 80 amino acids in the intracellular loop 3. In this report we show that the $hH_3R(365)$ is differentially expressed compared to the $hH_3R(445)$ and has a higher affinity and potency for H_3R agonists and conversely a lower potency and affinity for H_3R inverse agonists. Furthermore, we show a higher constitutive signaling of the $hH_3R(365)$ compared to the $hH_3R(445)$ in both guanosine-5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding and cAMP assays, likely explaining the observed differences in hH_3R pharmacology of the two isoforms. As H_3R ligands are beneficial in animal models of obesity, epilepsy and cognitive diseases such as Alzheimer's disease and attention deficit hyperactivity disorder (ADHD) and currently entered clinical trails, these differences in H_3R pharmacology of these two isoforms are of great importance for a detailed understanding of the action of H_3R ligands.

Introduction

The histamine H_3 receptor (H_3R) was originally discovered in the brain on histaminergic neurons as a presynaptic autoreceptor regulating the release of histamine (Arrang et al., 1983). Subsequently, the H_3R was found to regulate the release of other neurotransmitters, such as acetylcholine, dopamine, glutamate, noradrenalin, and serotonin (Schlicker et al., 1988; Schlicker et al., 1989; Clapham and Kilpatrick, 1992; Schlicker et al., 1993; Brown and Reymann, 1996). The histamine-containing cell bodies, located in the tuberomammillary nucleus of the posterior hypothalamus, project to most cerebral areas in rodent and human brain (Panula et al., 1984; Watanabe et al., 1984). Brain histamine is involved in the regulation of numerous functions of the central nervous system (CNS), including arousal, cognition, locomotor activity, autonomic and vestibular functions, feeding and drinking, sexual behaviour, and analgesia (Hough, 1988; Schwartz et al., 1991; Wada et al., 1991). Moreover H_3R specific ligands show beneficial effects in animal models of obesity, epilepsy and cognitive diseases such as Alzheimer's disease and attention deficit hyperactivity disorder (ADHD) (Hancock, 2003; Passani et al., 2004; Leurs et al., 2005). Consequently, H_3R antagonists are considered as potential new therapeutics and are currently undergoing clinical trails (Celanire et al., 2005).

For a good understanding of the biological effects of H_3R ligands a detailed knowledge of the molecular pharmacology of the human H_3R (hH_3R) is

indispensable. The molecular cloning of the hH_3R (Lovenberg et al., 1999) has revealed that the hH_3 gene is located on chromosome 20 (20q13.32-20q13.33) and contains three introns (Cogé et al., 2001), which give rise to a large number of hH_3R isoforms through alternative splicing (for reviews see Hancock et al., 2003; Leurs et al., 2005). At present, there is no detailed knowledge on the pharmacological consequences of the various alternative splicing events. On the basis of the currently published information, one can conclude that the $hH_3R(365)$ is one of the most abundantly expressed hH_3R isoforms next to the full length $hH_3R(445)$ (Cogé et al., 2001; Wellendorph et al., 2002; Esbenshade et al., 2006). The $hH_3R(365)$ isoform lacks 80 amino acids in the third intracellular loop (IL3) and has been described to be non-functional in a cAMP, guanosine-5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding and Ca^{2+} mobilization assay (Cogé et al., 2001), but to be functional in a R-SATTM reporter assay (Wellendorph et al., 2002). For other G-protein coupled receptors (GPCRs), the IL3 has been shown to dictate G-protein specificity (Burstein et al., 1996; Senogles et al., 2004), bind β-arrestins (Gelber et al., 1999) and calmodulin (Turner et al., 2004). Furthermore, the carboxyl terminus of the IL3 has been shown to play a role in constitutive activity of GPCRs (Chakir et al., 2003). In view of the relative high abundance of the $hH_3R(365)$ isoform and the known importance of the IL3 loop in GPCR mediated signal transduction, we pharmacologically characterized the $hH_3R(445)$ and the $hH_3R(365)$ isoforms in full detail in this study.

Results

Differential expression in the central nervous system

The relative expression of the $hH_3R(445)$ and $hH_3R(365)$ isoforms was assessed by RT-PCR in several areas of the human CNS. For the $hH_3R(445)$ high expression was found in the cerebellum and the caudate, moderate expression was found in the hypothalamus, cerebrum and the thalamus (Figure 1). Low expression was found in all other tested regions and very low expression was found in the spinal cord. In most of the tested regions the $hH_3R(365)$ was found to be approximately 1.4-fold higher expressed than the $hH_3R(445)$. The biggest differences in expression for the two isoforms was found in the regions were the $hH_3R(445)$ was higher expressed than the $hH_3R(365)$, such as the caudate (3.5-fold), corpus callosum (2.8-fold) and the spinal cord (2.2-fold).

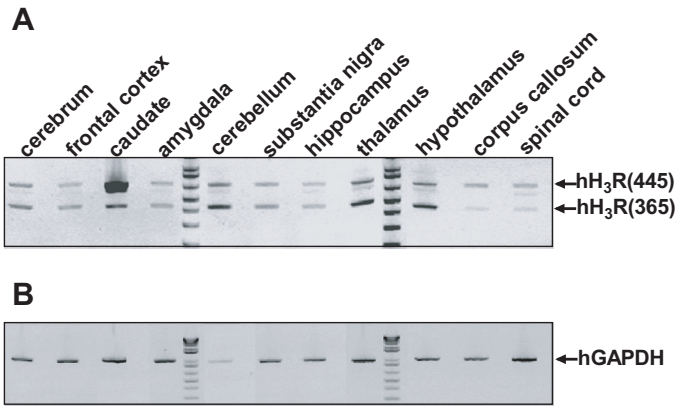

Figure 1. Expression of the $hH_3R(445)$ and the $hH_3R(365)$ isoforms identified in the human central nervous system by RT-PCR. (A, B) mRNA of human brain tissues was amplified by RT-PCR using primers flanking the intronic region. PCR products were analyzed using agarose gels stained with ethidium bromide. The lengths of amplicons were estimated by molecular mass markers. (B) Human GAPDH amplification was used as an internal standard.

Saturation analysis reveals distinct populations for the hH_3R isoforms

The hH_3R isoforms (445 and 365) stably transfected into rat glioma C6 cells were characterized by saturation analysis with the agonist N^{α}-[methyl-^{3}H]-histamine and with the inverse agonists [^{125}I]-Iodophenpropit (Jansen et al., 1994) and [^{3}H]A-349821 {4'-[3-((2R,5R)-2,5-Dimethyl-pyrrolidin-1-yl)-propoxy]-biphenyl-4-yl}-morpholin-4-yl-methanone (Witte et al., 2006). Saturation binding experiments with N^{α}-[methyl-^{3}H]-histamine revealed a single high affinity binding site for both isoforms with a similar maximal number of binding sites ($hH_3R(445)$: B_{max} = 630 ± 100 fmol/mg protein, n=7 and $hH_3R(365)$: B_{max} = 670 ± 40 fmol/mg protein, n=4). Yet, for the radioligand N^{α}-[methyl-^{3}H]-histamine a significantly lower affinity (p<0.001) was measured for the $hH_3R(445)$ compared to the $hH_3R(365)$ (K_d values of 0.81 ± 0.07 nM (n=7) and 0.33 ± 0.04 nM (n=5) were obtained for the $hH_3R(445)$ and $hH_3R(365)$ respectively; see Figures 2A and 2D). Both hH_3R isoforms exhibited similar affinities for [^{125}I]-Iodophenpropit ($hH_3R(445)$: K_d = 3.2 ± 2 nM and $hH_3R(365)$: K_d = 3.1 ± 2 nM), but a significant difference (p<0.05) was found for the maximal number of binding sites ($hH_3R(445)$: B_{max} = 2000 ± 500 fmol/mg protein, n=3 and $hH_3R(365)$ and B_{max} = 830 ± 20 fmol/mg protein, n=3) (Figures 2A and 2D). As observed for [^{125}I]-Iodophenpropit, the affinity values for the H_3R inverse agonist [^{3}H]A-349821 did not differ between the two isoforms ($hH_3R(445)$: K_d = 0.081 ± 0.01 nM, n=4 and $hH_3R(365)$: K_d = 0.10 ± 0.02 nM, n=4), but a significant difference was again found for the maximal number of binding sites ($hH_3R(445)$:

B_{max} = 1800 ± 100 fmol/mg protein, n=4 and $hH_3R(365)$ and B_{max} = 780 ± 100 fmol/mg protein, n=4) (Figures 2C and 2D).

G-protein uncoupling affects N^{α}-[methyl-^{3}H]-histamine binding to $hH_3R(445)$

To study the effect of G-proteins on the binding characteristics of the two hH_3R isoforms, saturation binding experiments were performed under conditions that would prevent G-protein coupling. Co-incubation of 0.1 mM GDP in the saturation binding experiment resulted in a significant 2.7-fold inhibition (p<0.05) of the maximal number of binding sites of N^{α}-[methyl-^{3}H]-histamine to $hH_3R(445)$ expressing membranes, but had no effect on the binding to $hH_3R(365)$ expressing membranes. The affinity of both isoforms for N^{α}-[methyl-^{3}H]-histamine was unaltered in the presence of 0.1 mM GDP ($hH_3R(445)$: K_d = 1.2 ± 0.05 nM, n=3 and $hH_3R(365)$ K_d= 0.4 ± 0.09 nM, n=3). In $hH_3R(445)$ or $hH_3R(365)$ expressing membranes neither the maximal number of binding sites nor the affinity for [^{3}H]A-349821 was affected by co-incubation of 0.1 mM GDP (Figure 2E). In homologous competition experiments with N^{α}-[methyl-^{3}H]-histamine at $hH_3R(445)$ expressing membranes co-incubation with GDP, GTPγS or pretreatment with 200 ng/ml PTX for 16h all had a similar effect on the maximal specific binding of N^{α}-[methyl-^{3}H]-histamine. None of these treatments had an effect on the maximal specific binding of N^{α}-[methyl-^{3}H]-histamine to $hH_3R(365)$ expressing membranes (Figure 2F).

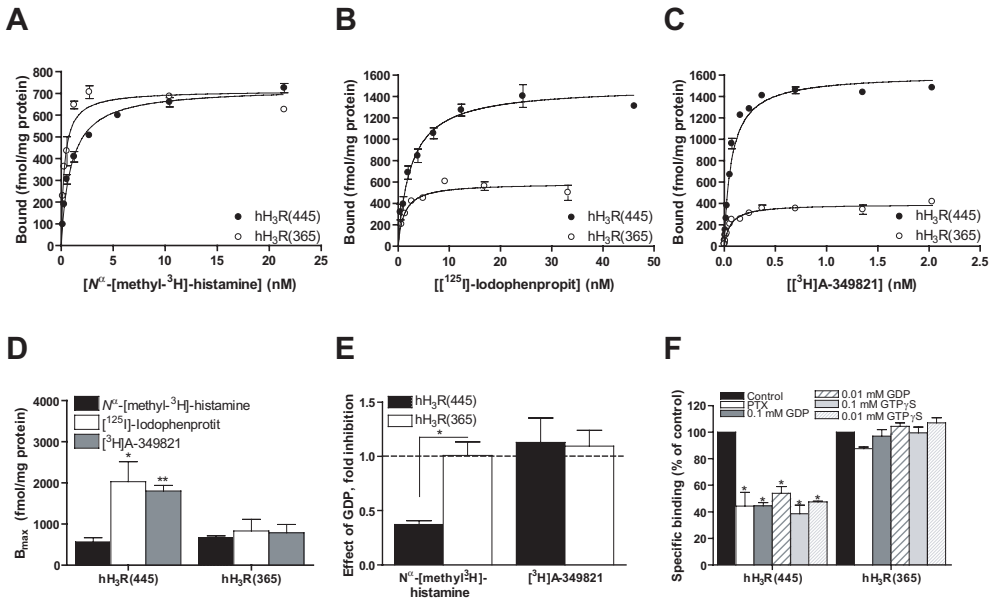

Figure 2. Typical saturation curves for (A) N^{α}-[methyl-^{3}H]-histamine, (B) [^{125}I]-Iodophenpropit and (C) [^{3}H]A-349821 on membranes of C6 cells expressing either the $hH_3(445)$ (●) or the $hH_3(365)$ (○). Non-specific binding was determined in the presence of 100 μM thioperamide. (D) Maximal specific binding (B_{max}) determined with N^{α}-[methyl-^{3}H]-histamine, [^{125}I]-Iodophenpropit and [^{3}H]A-349821 at $hH_3(445)$ and $hH_3(365)$ expressing membranes. * p<0.05, ** p<0.01 *versus* $hH_3R(365)$. (E) Relative effects of co-incubation of 0.1 mM Guanosine 5′-diphosphate (GDP) on the maximal specific binding of membranes of C6 cells expressing the $hH_3R(445)$ or $hH_3R(365)$ as determined with N^{α}-[methyl-^{3}H]-histamine or [^{3}H]A-349821. For each condition, the control samples without 0.1 mM GDP are set to one, indicated by the dashes line. * p<0.05 *versus* $hH_3R(365)$. (F) Effects of various agents (Pertussis toxin (PTX), guanosine 5′-diphosphate (GDP) and guanosine 5′-[γ-thio]triphosphate (GTPγS) that uncouple the receptor from the G-protein on the specific binding in a homologous competition experiment. * p<0.05 versus control. Results represent the mean ± S.E.M of a typical experiment (A-C), or the mean ± S.E.M. of at least three independent experiments performed in triplicate (D-F).

Pharmacological profile of radioligand binding to the $hH_3R(445)$ and $hH_3R(365)$ isoforms

A series of imidazole (compounds 1-14 and 22-28) and non-imidazole (compounds 15-21) containing H_3R ligands were subsequently tested in a heterologous competitive binding assay with N^{α}-[methyl-^{3}H]-histamine and [^{125}I]-Iodophenpropit with membranes expressing either the $hH_3R(445)$ or the $hH_3R(365)$ (see Table 1). The competition binding curves for all compounds were best fitted according to a single binding site model and showed to have Hill-slopes close to unity for both

radioligands (Figures 3A-D). Equilibrium dissociation constants of the agonists correlate highly for competitive binding with either N^{α}-[methyl-^{3}H]-histamine (r^2=0.82) or [^{125}I]-Iodophenpropit (r^2=0.85) (Figures 3E and 3F). Agonists exhibit a higher affinity for $hH_3R(365)$ compared to $hH_3R(445)$, on average 3.4-fold or 55-fold when determined with N^{α}-[methyl-^{3}H]-histamine or [^{125}I]-Iodophenpropit respectively (Figure 3E). Equilibrium dissociation constants of the inverse agonists correlate highly as well (r^2=0.95 for N^{α}-[methyl-^{3}H]-histamine and r^2=0.91 for [^{125}I]-Iodophenpropit), but display an opposite preference for the hH_3R isoforms and have a 2-3-fold higher affinity for the $hH_3(445)$ isoform (Figure 3F).

In the correlation plots from the heterologous competitive binding assay with N^{α}-[methyl-^{3}H]-histamine or [^{125}I]-Iodophenpropit some compounds are outside the 95% confidence interval of the linear regression for both the agonist and inverse agonist and therefore are statistical outliers (Figures 3E and 3F).

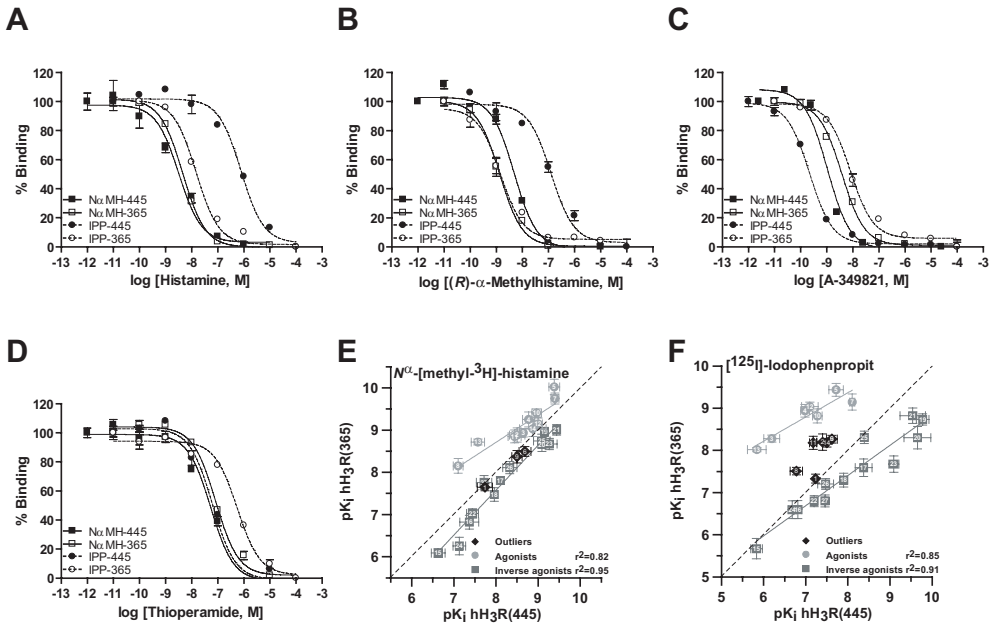

Figure 3. Competition binding experiment curves and corresponding correlation plots. (A-D) Competition binding curves determined with 0.8 nM N^{α}-[methyl-^{3}H]-histamine (NαMH) or 2 nM [^{125}I]-Iodophenpropit (IPP) in the presence of various concentrations of H_3R compounds in membranes of C6 cells stably expressing the $hH_3R(445)$ or the $hH_3R(365)$. Results represent the mean ± S.E.M of a typical experiment. (E, F) Affinity correlation plots for a series of selective H_3R agonists (●, compounds 1-14) and inverse agonists (■, compounds 15-28) determined in radioligand competition binding experiments with either N^{α}-[methyl-^{3}H]-histamine (E) or [^{125}I]-Iodophenpropit (F) on membranes expressing the $hH_3R(445)$ or the $hH_3R(365)$. A good agreement between binding affinities is observed when agonists and inverse agonists considered as a different group. Compounds outside the 95% confidence interval for both groups are indicated as outliers (♦). Unity is indicated by the dashed line.

Table 1. Affinity values from competition experiments using N^{α}-[methyl-^{3}H]-histamine or [^{125}I]-Iodophenpropit in membranes expressing $hH_3R(445)$ or $hH_3R(365)$. Affinities are expressed as mean pKi ± SEM (n=3-9).

#	Compound	N^{α}-[methyl-^{3}H]-histamine						[^{125}I]-Iodophenpropit					
		$hH_3R(445)$			$hH_3R(365)$			$hH_3R(445)$			$hH_3R(365)$		
1	FUB322	7.7	±	0.2	7.6	±	0.1	7.2	±	0.0	7.3	±	0.1
2	GT-2331	8.5	±	0.2	8.9	±	0.2	7.4	±	0.1	8.2	±	0.2
3	Histamine	8.6	±	0.0	8.9	±	0.1	6.2	±	0.2	8.3	±	0.1
4	Imbutamine	8.8	±	0.1	9.3	±	0.2	7.0	±	0.10	8.9	±	0.1
5	Imetit	9.4	±	0.1	10	±	0.2	7.7	±	0.2	9.4	±	0.2
6	Imhexamine	8.7	±	0.1	8.5	±	0.1	7.3	±	0.0	7.7	±	0.2
7	Immepip	9.4	±	0.1	9.7	±	0.1	8.1	±	0.1	9.1	±	0.2
8	Imoproxifan	7.1	±	0.1	8.2	±	0.2	6.8	±	0.1	7.5	±	0.1
9	Impentamine	8.5	±	0.1	8.4	±	0.1	7.6	±	0.1	8.3	±	0.1
10	N^{α}-methylhistamine	9.0	±	0.1	9.4	±	0.1	7.3	±	0.1	8.8	±	0.2
11	Proxyfan	8.4	±	0.2	8.9	±	0.2	7.2	±	0.1	8.2	±	0.2
12	(*R*)-α-methylhistamine	9.0	±	0.1	9.1	±	0.1	7.1	±	0.2	9.0	±	0.1
13	(*S*)-α-methylhistamine	7.6	±	0.2	8.7	±	0.1	5.8	±	0.2	8.0	±	0.1
14	VUF5681	8.9	±	0.3	9.0	±	0.2	7.5	±	0.0	8.2	±	0.1
15	A-304121	6.6	±	0.2	6.1	±	0.1	5.8	±	0.1	5.7	±	0.2
16	A-317920	7.4	±	0.2	6.8	±	0.2	6.8	±	0.1	6.6	±	0.2
17	A-320436	8.1	±	0.1	7.8	±	0.1	8.4	±	0.2	7.6	±	0.2
18	A-331440	8.0	±	0.0	7.5	±	0.2	7.9	±	0.1	7.3	±	0.2
19	A-349821	9.2	±	0.1	9.0	±	0.1	9.8	±	0.1	8.7	±	0.1
20	A-358239	9.1	±	0.1	8.7	±	0.2	9.7	±	0.3	8.3	±	0.3
21	A-431404	9.4	±	0.1	9.0	±	0.1	9.5	±	0.3	8.8	±	0.2
22	Ciproxifan	7.4	±	0.1	7.0	±	0.1	7.2	±	0.1	6.8	±	0.1
23	Clobenpropit	9.3	±	0.2	8.7	±	0.1	9.1	±	0.1	7.7	±	0.2
24	GT-2016	7.1	±	0.1	6.3	±	0.2	6.7	±	0.1	6.6	±	0.2
25	Iodophenpropit	9.1	±	0.2	8.7	±	0.2	8.4	±	0.1	8.3	±	0.1
26	*i*-propyl-impentamine	8.3	±	0.2	8.1	±	0.1	7.5	±	0.2	7.2	±	0.1
27	Thioperamide	7.7	±	0.2	7.8	±	0.2	7.5	±	0.01	6.8	±	0.1

Pharmacological profile of the $hH_3R(445)$ and $hH_3R(365)$ induced [^{35}S]-GTPγS binding

A series of compounds was also tested in a functional [^{35}S]-GTPγS binding assay with membranes expressing either the $hH_3R(445)$ or the $hH_3R(365)$ isoform (see Table 2). The dose response curves for all compounds were best fitted according to a single binding site model and showed to have Hill-slopes close to unity (Figures 4A-E). Potencies of the agonists correlated highly (r^2=0.96) and were found to be, on average, 4.6-fold more potent at the $hH_3R(365)$ (Figure 4F).

However, the maximal increase in [^{35}S]-GTPγS binding for full agonists was approximately 80% lower at the hH_3(365) (1.2-fold increase of basal) compared to the hH_3R(445) (2.2-fold increase of basal) (Figure 4A-C).
Potencies of the tested inverse agonist correlate highly as well (r^2=0.99), but were found to be, on average, 2.6-fold less potent at the hH_3R(365) (Figure 4F). Interestingly, non-imidazole ligands, like A-349821 showed a significantly (p<0.01) higher inhibition of basal [^{35}S]-GTPγS binding for the hH_3R(365) compared to the hH_3R(445) (Figures 4D and 4E).

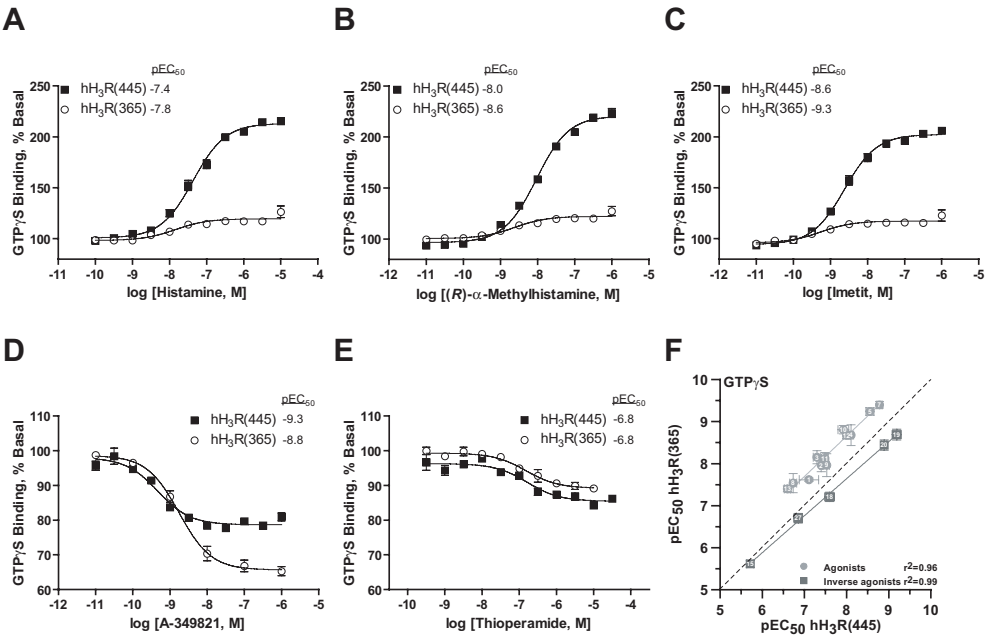

Figure 4. [^{35}S]-GTPγS binding curves and corresponding correlation plot. (A-E). Modulation of [^{35}S]-GTPγS binding by increasing concentrations of H_3R agonists and antagonists in membranes from C6 cells stably expressing the hH_3R(445) or the hH_3R(365). Data are expressed as a percentage of the basal [^{35}S]-GTPγS binding in the absence of ligands (basal activities are isoform and time dependent: (Panels A-C: hH_3R(445), 118 ± 3 fmol/mg; hH_3R(365), 220 ± 25 fmol/mg; Panels D-E: hH_3R(445), 354 ± 45 fmol/mg, hH_3R(365), 782 ± 72 fmol/mg). Results represent the mean ± S.E.M of a typical experiment. (F) Potency correlation plots for a series of selective H_3R agonists (●, 1-14) and inverse agonists (■, 15-28) determined in a [^{35}S]-GTPγS binding assay on membranes expressing the hH_3R(445) or the hH_3R(365). A good agreement between potencies is observed when agonists and inverse agonists considered as a different group. Unity is indicated by the dashed line.

Table 2. Potency values from [^{35}S]-GTPγS binding in membranes expressing $hH_3R(445)$ or $hH_3R(365)$. Potencies are expressed as mean pEC_{50} ± SEM (n=3-7). (*R*)-α-methylhistamine α=1 and ABT-239 α=-1 by definition.

#	Compound	$hH_3R(445)$						$hH_3R(365)$					
		pEC_{50}			α			pEC_{50}			α		
1	FUB322	7.1	±	0.2	0.15	±	0.002	7.6	±	0.0	0.56	±	0.01
2	GT-2331	7.4	±	0.0	0.58	±	0.004	8.0	±	0.1	0.71	±	0.02
3	Histamine	7.3	±	0.1	0.89	±	0.001	8.2	±	0.2	0.92	±	0.01
4	Imbutamine	8.1	±	0.1	0.80	±	0.01	8.7	±	0.2	0.57	±	0.01
5	Imetit	8.6	±	0.1	0.81	±	0.004	9.2	±	0.1	0.87	±	0.01
6	Imhexamine	7.2	±	0.1	0.13	±	0.003		-		0.0		
7	Immepip	8.8	±	0.1	0.77	±	0.003	9.4	±	0.1	0.79	±	0.01
8	Imoproxifan	6.7	±	0.1	0.49	±	0.003	7.5	±	0.2	0.59	±	0.01
9	Impentamine	7.5	±	0.1	0.31	±	0.002	8.0	±	0.3	0.44	±	0.01
10	N^{α}-methylhistamine	7.9	±	0.1	1.0	±	0.03	8.8	±	0.1	0.99	±	0.05
11	Proxyfan	7.5	±	0.1	0.66	±	0.01	8.1	±	0.1	0.83	±	0.01
12	(*R*)-α-methylhistamine	8.0	±	0.1	1.0	±	0.01	8.7	±	0.1	1.0	±	0.01
13	(S)-α-methylhistamine	6.6	±	0.1	0.94	±	0.01	7.4	±	0.1	1.14	±	0.01
15	A-304121	5.7	±	0.1	-0.87	±	0.01	5.6	±	0.1	-0.66	±	0.01
18	A-331440	7.6	±	0.1	-0.88	±	0.03	7.2	±	0.1	-1.0	±	0.01
19	A-349821	9.2	±	0.1	-0.93	±	0.01	8.7	±	0.1	-1.1	±	0.01
20	A-358239	8.9	±	0.1	-1.0	±	0.01	8.4	±	0.1	-1.0	±	0.01
27	Thioperamide	6.9	±	0.1	-0.52	±	0.01	6.7	±	0.1	-0.38	±	0.01

Pharmacological profile of the forskolin-induced cAMP levels by the $hH_3R(445)$ and the $hH_3R(365)$

Histamine H_3R specific compounds were also tested for their modulation of cAMP levels in forskolin (0.5 μM) stimulated C6 cells expressing either the $hH_3R(445)$ or the $hH_3R(365)$ (see Table 3). All tested agonists and inverse agonists potencies correlate highly (r^2=0.91 and r^2=0.88, respectively). Agonists are approximately 35-fold more potent at the $hH_3R(365)$ (Figure 5F). Similar to the [^{35}S]-GTPγS binding assay, full agonist are however more efficacious at the $hH_3R(445)$ (80% and 44% inhibition of the forskolin induced cAMP production, respectively; see Figures 5A-C). Conversely, inverse agonist are approximately 14-fold less potent at the $hH_3R(445)$, but appear more efficacious (Figures 5D and E). Especially, non-imidazole compounds, like A-349821, showed increased efficacy at the $hH_3R(365)$ (3.0-fold increase over basal) compared to the $hH_3R(445)$ (1.5-fold increase over basal) (Figure 5D).

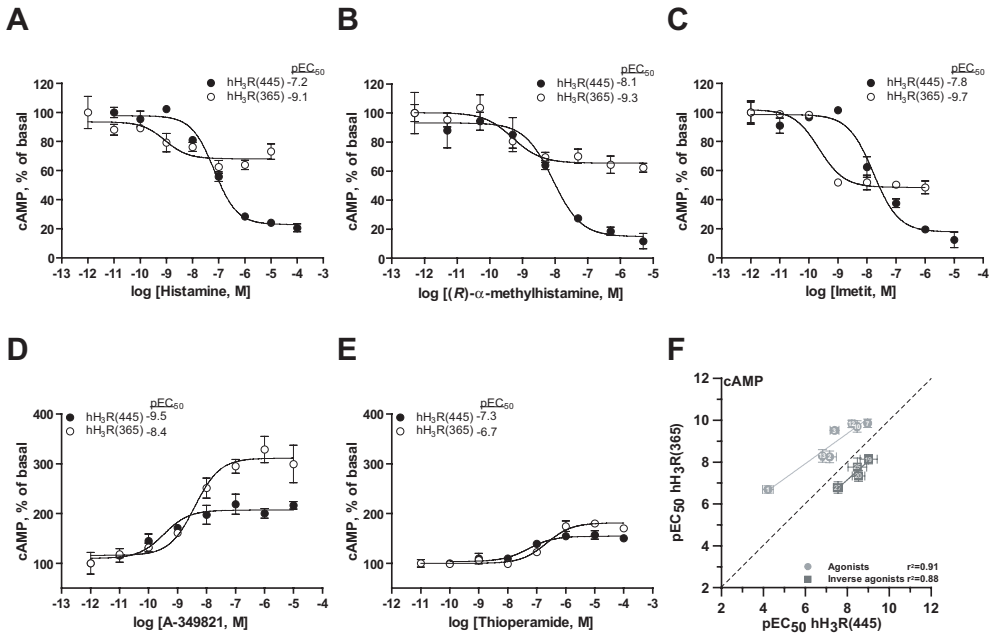

Figure 5. cAMP formation curves and corresponding correlation plot. (A-E) Modulation of 0.5 μM forskolin-stimulated cAMP formation by increasing concentrations of H_3R ligands in C6 cells stably expressing the $hH_3R(445)$ or the $hH_3R(365)$. Data are expressed as a percentage of the basal cAMP levels in the absence of ligands. Results represent the mean ± S.E.M of a typical experiment. (F) Potency correlation plots for a series of selective H_3R agonists (●, compounds 1-14) and inverse agonists (■, compounds 15-28) determined in a cAMP formation assay on C6 cells expressing the $hH_3R(445)$ or the $hH_3R(365)$. A good agreement between potencies is observed when agonists and inverse agonists considered as a different group. Unity is indicated by the dashed line.

Table 3. Potency values from a cAMP formation assay in C6 cells expressing $hH_3R(445)$ or $hH_3R(365)$. Potencies are expressed as mean pEC50 SEM (n=2-7). (*R*)-α-methylhistamine α=1 and ABT-239 α=-1 by definition.

#	Compound	$hH_3R(445)$						$hH_3R(365)$					
		pEC_{50}			α			pEC_{50}			α		
1	FUB322	4.2	±	0.3	1.2	±	0.2	6.7	±	0.2	1.1	±	0.04
2	GT-2331	7.2	±	0.3	0.76	±	0.1	8.2	±	0.3	1.0	±	0.1
3	Histamine	7.4	±	0.2	1.0	±	0.02	9.5	±	0.1	1.0	±	0.1
7	Immepip	9.0	±	0.1	0.87	±	0.1	9.9	±	0.2	0.92	±	0.1
10	N^{α}-methylhistamine	8.5	±	0.1	1.1	±	0.01	9.7	±	0.3	1.0	±	0.2
12	(R)-α-methylhistamine	8.2	±	0.1	1.0	±	0.03	9.8	±	0.2	1.0	±	0.1
13	(S)-α-methylhistamine	6.8	±	0.1	1.1	±	0.0	8.3	±	0.3	1.0	±	0.2
19	A-349821	9.0	±	0.4	-1.0	±	0.1	8.1	±	0.2	-1.0	±	0.2
20	A-358239	8.5	±	0.3	-0.90	±	0.1	7.3	±	0.3	-1.1	±	0.1
25	Iodophenpropit	8.5	±	0.4	-0.77	±	0.05	7.8	±	0.4	-0.50	±	0.1
27	Thioperamide	7.6	±	0.2	-0.83	±	0.1	6.8	±	0.3	-0.71	±	0.05

The $hH_3R(365)$ is more constitutively active than the $hH_3R(445)$

The observed differences in agonist/inverse agonist efficacies are good indications of substantial differences in the levels of constitutive activity of the two hH_3R isoforms. To investigate this issue, the amount of [^{35}S]-GTPγS formed over a time-course of 10 minutes was measured on C6 cell membranes expressing either the $hH_3R(445)$ or the $hH_3R(365)$. The C6 parental cell line showed a gradual increase in the amount [^{35}S]-GTPγS bound and no modulation by the specific H_3R ligands (*R*)-α-methylhistamine and A-349821 was observed (Figure 6A). Basal increase in [^{35}S]-GTPγS binding in C6 cells expressing the $hH_3R(445)$ was comparable to the parental cell line, whereas for the $hH_3R(365)$ the basal increase was significantly higher (Figure 6A-D). Stimulation of the $hH_3R(445)$ with the H_3R agonist (*R*)-α-methylhistamine led to a steep increase in [^{35}S]-GTPγS binding over time, while the inverse agonist, A-349821 slightly inhibited the [^{35}S]-GTPγS binding (Figure 6B). On membranes expressing the $hH_3R(365)$, the increase in [^{35}S]-GTPγS binding induced by (*R*)-α-methylhistamine was moderate, whereas the effect of A-349821 was more pronounced compared to its effect at the $hH_3R(445)$ (Figures 6A-D).

Also forskolin (0.5 μM) induced cAMP levels were 2.3-fold (p<0.001) lower in C6 cells expressing the $hH_3R(365)$ compared to C6 cells expressing $hH_3R(445)$ (Figure 6E). The H_3R agonist (*R*)-α-methylhistamine inhibited the forskolin (0.5 μM) induced to a similar absolute level in both $hH_3R(445)$ or $hH_3R(365)$ expressing C6 cells. Likewise, stimulation of C6 cells expressing either the $hH_3R(445)$ or the $hH_3R(365)$ with the H_3R inverse agonist A-349821 increased the cAMP levels to a similar absolute level of cAMP.

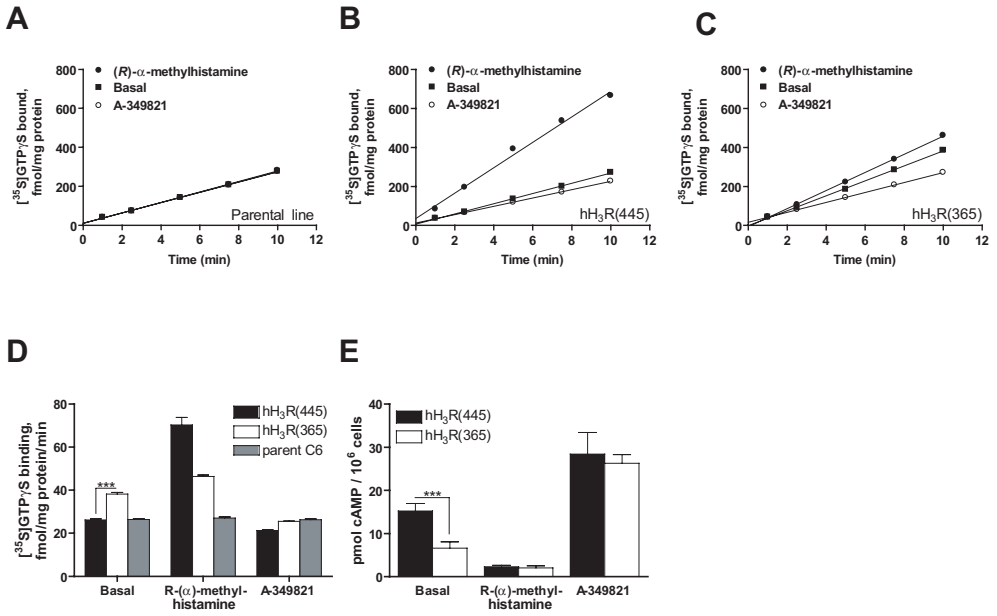

Figure 6. Basal and H_3R ligand mediated [^{35}S]-GTPγS and cAMP signaling in C6 cells expressing the $hH_3R(445)$ or the $hH_3R(365)$. (A-C) Basal and ligand mediated [^{35}S]-GTPγS formed over a time-course of 10 minutes in parental C6 cells or C6 cells expressing either the $hH_3R(445)$ or $hH_3R(365)$. Total [^{35}S]-GTPγS formed in 10 minutes (fmol/mg protein) in the parental C6 cells and expressing the $hH_3R(445)$ or the $hH_3R(365)$. (D-E) Basal and ligand (1 μM (R)-α-methylhistamine or 0.1 μM A-349821) H_3R-mediated modulation of GTPγS binding (fmol/mg protein/minute) and forskolin (0.5 μM) induced cAMP levels in C6 cells expressing either the hH_3R (445) or the hH_3R (365).

Application of the cubic ternary model to explain the observed properties of the $hH_3R(365)$ and $hH_3R(445)$

We used the cubic ternary complex (CTC) model (Weiss et al., 1996a; Weiss et al., 1996b) to examine if the observed differences in affinities and potencies, the maximal number of binding sites of agonist and inverse agonist and the effect of GDP on the maximal number of binding sites, could all be explained by an increase in GPCR constitutive activity, represented in the CTC model by the equilibrium constant of the receptor (K_{act}; see Figure 7A).

To examine the maximal number of binding site as found with the H_3R radioligands (Figure 2A), the agonist radioligand N^{α}-[methyl-^{3}H]-histamine was assumed to bind the R_a and R_aG state of the receptor, to promote receptor activation (α>1) and to facilitate G-protein coupling only for the receptor in its active form (δ>1). The inverse agonist radioligand [^{3}H]A-349821 was assumed to bind the R_i and R_iG state of the receptor, to inhibit receptor activation (α<1) and to facilitate G-protein

coupling only for the receptor in its inactive form (δ<1). Furthermore, to examine if the difference between the two isoforms could be due to a difference in K_{act}, the $hH_3R(445)$ and $hH_3R(365)$ were assumed to have a K_{act}<1 and K_{act}=1, respectively. Hereby, the $hH_3R(445)$ exists predominantly in the R_i conformation and the $hH_3R(365)$ has no preference for either the R_a of R_i conformation. Experimentally we found an approximately 3-fold difference in maximal number of binding sites of [^{3}H]A-349821 for the two hH_3R isoforms (Figure 2D). To take this observation into account in the CTC-model, the R_{tot} of the $hH_3R(365)$ was assumed to be 3-fold lower compared to the $hH_3R(445)$.

Subsequently, the maximal number of binding sites for the agonist (NαMH) and inverse agonist (IPP) were simulated in the presence (G_{tot}=1) and absence (G_{tot}=0) of G-proteins. This simulation revealed that only the maximal number of binding sites for the agonist, reflected by the receptor states LR_a and LR_aG, on the $hH_3R(445)$ is affected by the removal of G-proteins (Figure 7B). Furthermore, the maximal number of binding sites for the $hH_3R(445)$ recognized by the inverse agonist, reflected by the receptor states LR_i and LR_iG, was found to be approximately three times higher, whereas for the $hH_3R(365)$ agonist and inverse agonists give rise to the same maximal number of binding sites (Figure 7B).

To account for the differences in ligand affinity and potency found experimentally for the $hH_3R(445)$ and $hH_3R(365)$, we calculated the apparent affinity constant when G-proteins are not limiting (K_{Aapp}) (Weiss et al., 1996b).

$$K_{Aapp} = \frac{\gamma K_A (1 + \alpha\beta\delta K_{act})}{1 + \beta K_{act}} \quad (1)$$

From this equation (Eq. 1) it follows that for the $hH_3R(445)$ the $\log(K_{Aapp})$ for agonists is 10.2 and for inverse agonists 10.0, in case of the $hH_3R(365)$ the $\log(K_{Aapp})$ was increased for agonists to 11.0 and decreased for inverse agonists to 9.0. We defined a partial agonist to have a α>1, but lower than α of a full agonist, e.g. the ligand is able to activate the receptor, but to a lesser extent than a full agonist. The difference for partial agonists in the K_{Aapp} for the two isoforms decreases and with a $\alpha \approx 1$ the $\log(K_{Aapp})$=10 for both the $hH_3R(445)$ and the $hH_3R(365)$.

Furthermore, we simulated radioligand competition and dose-response curves for the $hH_3R(445)$ and $hH_3R(365)$ in a G-protein dependent manner (Monczor et al., 2003). For the competition curves we assumed the radioligands and the competing ligands to have properties as described above. Subsequently, the concentration of a ligand (L) was varied and the generated curves were plotted as percentage of the B_{max} for both hH_3R isoforms and radioligands (Figure 7C and D).

For simulation of dose-response curves we assumed that both the LR_aG and R_aG give rise to a receptor response (as described by Chen et al., 2000). Subsequently,

the following equation (Eq. 2) was used to simulate the dose-response curves (Figure 7E).

$$\rho = \frac{\beta K_{act}[R_i]K_G(1+\delta\alpha\gamma[L]K_A)}{[L]K_A(\gamma[R_i]*K_G(1+\delta\alpha\beta K_{act}))+1+R_iK_G(1+\beta K_{act})} \quad (2)$$

Furthermore, when [L]=0, the above equation describes the basal signaling of the receptor and this was found to be 19-fold increased for the $hH_3R(365)$ compared to the $hH_3R(445)$.

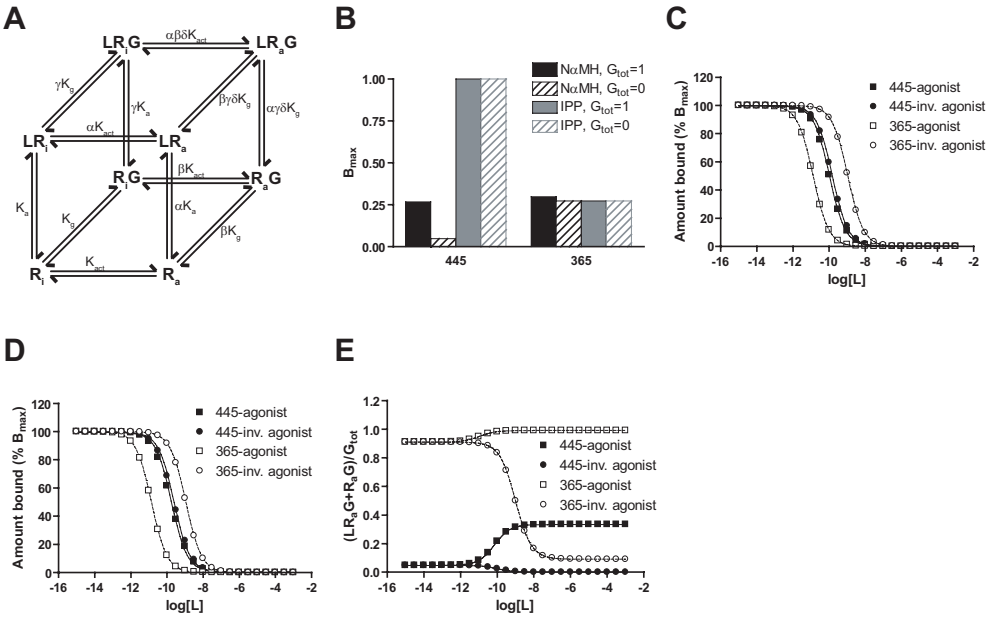

Figure 7. Computer simulations according to the CTC-model. (A) CTC-model with equilibrium association constants (Adapted from Weiss et al., 1996a; Weiss et al., 1996b) in which; K_a and K_g represent the association constants for a ligand (L) and G-protein (G), respectively; K_{act} is the equilibrium constant for the activation of the receptor (R); α and γ represent the effect of ligand binding on activation and G-protein coupling of the receptor, respectively; β represent the effect of receptor activation on G-protein coupling and δ represents the extent to which the joint effect of any two (receptor activation, G-protein coupling or ligand binding) varies the level of the third. (B) Theoretical maximal specific binding of an agonist (NαMH) or inverse agonist (IPP) in the presence (G_{tot}=1) or absence (G_{tot}=0) of G-proteins. (C and D) Theoretical radioligand competition of an agonist (C) or inverse agonist (D) radioligand by an unlabeled agonist or inverse agonists on the $hH_3R(445)$ and $hH_3R(365)$. Curves were plotted as a percentage of the calculated B_{max}. (E) Theoretical dose-response curve for the $hH_3R(445)$ and $hH_3R(365)$ for agonists and inverse agonists. All simulations were generated with the following parameters: for agonists α=10 and δ=1, for inverse agonists α=0.1 and δ=0.1; for the $hH_3R(445)$ K_{act}=0.005 and R_{tot}=1 and for the $hH_3R(365)$ K_{act}=1 and R_{tot}=0.3. The other parameters were kept constant (β=10, γ=10, K_a=K_G=10^9, G_{tot}=1).

Discussion

The cloning of the $hH_3R(445)$ by Lovenberg et al. in 1999 (Lovenberg et al., 1999) has subsequently led to the discovery of a large number of hH_3R isoforms (Hancock et al., 2003; Leurs et al., 2005). In this study, we pharmacologically characterize the two most abundantly occurring hH_3R isoforms, the $hH_3R(445)$ and

the $hH_3R(365)$ (Cogé et al., 2001; Wellendorph et al., 2002; Esbenshade et al., 2006b). Using a RT-PCR approach we confirmed the abundant expression of the $hH_3R(445)$ and $hH_3R(365)$ isoforms and demonstrated differential isoform expression in various human brain areas. In most regions the $hH_3R(365)$ was slightly higher expressed. However, in brain regions with the highest $hH_3R(445)$ expression levels, such as caudate, corpus callosum and the spinal cord, the largest differences between these two isoforms was found.

The $hH_3R(365)$ lacks 80 amino acids in the IL3 in comparison to the $hH_3R(445)$ and was found to have different functionality. In particular, we show that the $hH_3R(365)$ isoform stably expressed in C6 cells gives rise to a higher agonist-independent inhibition of forskolin induced cAMP levels and to an increased basal [^{35}S]-GTPγS formation. These findings are consistent with an increased constitutive receptor activity of the $hH_3R(365)$ compared to the $hH_3R(445)$. Basal GPCR signaling highly depends on the receptor expression levels, but is also regulated by the expression levels of its cellular signaling partners (Milligan, 2003). However, the observed increase in constitutive signaling of the $hH_3R(365)$ cannot be explained by a higher level of receptor expression, as it was found to have either the same (N^{α}-[methyl-^{3}H]-histamine) or lower ([^{3}H]A-349821 or [^{125}I]-Iodophenpropit) number of radioligand binding sites as observed for the $hH_3R(445)$. Additionally, both isoforms were stably expressed using the same parental C6 cell line and differences in cellular content, like G-protein expression, are therefore unlikely.

Previously, agonist induced activation of the $hH_3R(365)$ was not found in several signal transduction assays, like the $G\alpha_i$-mediated inhibition of cAMP formation, [^{35}S]GTPγS binding or Ca^{2+} mobilization (Cogé et al., 2001), but in another study the $hH_3R(365)$ could be activated by agonists in a reporter assay (Wellendorph et al., 2002). In the present study we show that under our experimental conditions H_3R agonists can activate the $hH_3R(365)$ isoform resulting in an increase in [^{35}S]GTPγS binding and the expected inhibition of forskolin induced cAMP. The apparent discrepancy between the results of Coge et al. and the presents study might be due to the high constitutive activity of the $hH_3R(365)$, which makes it difficult to measure agonist mediated responses. Interestingly, the study of Coge et al. does not report on the functional effects of H_3R inverse agonists on the $hH_3R(365)$.

The $hH_3R(365)$ displays higher potencies and affinities for agonists and likewise lower potencies and affinities for inverse agonists, consistent with an increase constitutive activity. These findings are in line with previous observations with the rat H_3R_B and rat H_3R_C isoforms, which have a 32 or a 48 amino acid deletion in the IL3 loop respectively and also display a slightly higher affinity and potency for agonists compared to the full length rat H_3R_A isoform (Morisset et al., 2000; Drutel

et al., 2001). Interestingly, partial agonists have a higher affinity and potency at the $hH_3R(365)$ as well, but to a less extent than full agonists. The observed statistical outliers in the binding affinity correlation plots, in fact appear to be partial agonists with intrinsic activities between 0.0-0.4 or 0.0-0.7 in the correlation plots for N^{α}-[methyl-^{3}H]-histamine or [^{125}I]-Iodophenpropit respectively (Figures 3E and F, and Table 3). This is in good agreement with the CTC-model simulations, were we found that the α of a compound correlates with the difference in apparent affinity (K_{Aapp}) for the two isoforms, e.g. a decrease in α leads to a smaller difference in K_{Aapp} for the two hH_3R isoforms. Interestingly, agents that prevent G-protein coupling were found to affect the maximal number of binding sites of N^{α}-[methyl-^{3}H]-histamine only for the $hH_3R(445)$, but not for the $hH_3R(365)$. In the present study, the binding of the inverse agonists was unaffected by a treatment that would uncouple the receptor from the G-protein. This seems in contrast with earlier studies (Witte et al., 2006), but is most likely due to increased ionic strength of the incubation buffers, which is known to shift the equilibrium of the receptor to the inactive conformation (Costa et al., 1990). To account for these observations the CTC-model was used to examine if a higher K_{act} of a constitutively active receptor, might be able to explain these observations. Indeed, the CTC-model can account for the observed different maximal number of binding sites for the different radioligands and confirmed that the number of agonist labeled sites was affected only at the $hH_3R(445)$ when $[G_{tot}]=0$. Furthermore, an increase of the K_{act} resulted in the experimentally observed increase of potency and affinity of agonists and decreased potency and affinity of inverse agonists at the $hH_3R(365)$ compared to the $hH_3R(445)$. The CTC-model also accounted for the intermediate shift in affinity of partial agonists, showing that the preference over the $hH_3R(365)$ is dependent on intrinsic activity of the compounds. We used the CTC-model as other 'simpler' models, as the extended ternary complex (ECT) model, could not explain the effect of GDP. In this model, the removal of the G-protein affects predominately the receptor with the biggest K_{act}, which is not in accordance with our experimental observations.

In summary, our experimental and the CTC-modeling data indicate that the $hH_3R(365)$ isoform mainly functions in a constitutive manner. These data indicate that the 80 amino acid stretch in the third intracellular loop either imparts a negative constraint on the GPCR activation directly or is involved in the interaction of intracellular proteins that inhibit H_3R activation. Future studies should address the role of the 80 amino acid stretch in the IL3 on H_3R receptor function in more detail. Since the H_3R is one of the few examples for which GPCR constitutive activity has been shown to be prominent under *in vivo* conditions (Morisset et al., 2000; Drutel et al., 2001) our present results suggest that this effect might be mainly mediated

by the $hH_3R(365)$ isoform. The non-imidazole compounds show a higher negative intrinsic activity at the $hH_3R(365)$ compared to classical imidazole containing compounds like thioperamide. In combination with the potential differential expression in the CNS, this might lead to specific effects of these non-imidazole inverse agonists in brain areas where the $hH_3R(365)$ is higher expressed than the $hH_3R(445)$.

Materials and methods

Materials. Dulbecco's Modified Eagle Medium, trypsin-EDTA, penicillin, L-glutamine and streptomycin were from Invitrogen (Invitrogen, Breda, The Netherlands) and fetal calf serum was from Integro (Zaandam, The Netherlands). Culture dishes were from Costar (Haarlemermeer, The Netherlands). cyclic 3',5'-adenosine monophosphate (cAMP) was obtained from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). All H_3R ligands were (re-)synthesized at the Vrije Universiteit Amsterdam or at Abbott Laboratories. G-418 was obtained from Duchefa (Duchefa Biochemie B.V., Haarlem, The Netherlands). 3-isobutyl-1-methylxanthine was obtained from Acros (Fischer Scientific, 's Hertogenbosch, The Netherlands). N^{α}-[methyl-^{3}H]histamine (83 Ci/mmol) was from PerkinElmer Life Sciences (Zaventem, Belgium). [3H]cyclic 3',5'-adenosine monophosphate ([3H]cAMP, 40 Ci/mmol), was from Amersham ('s Hertogenbosch, The Netherlands). The following compound-abbreviations were used: FUB-322 for 3-(1H-imidazol-4-yl)propyl-di(p-fluorophenyl)-methyl ether hydrochloride, A-304121 for (R)-2-Amino-1-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-propan-1-one, A-317920 for Furan-2-carboxylic acid ((R)-2-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-1-methyl-2-oxo-ethyl)-amide, A-320436 for Furan-2-carboxylic acid ((R)-2-{4-[3-(4'-cyano-biphenyl-4-yloxy)-propyl]-[1,4]diazepan-1-yl}-2-oxo-1-thiazol-4-ylmethyl-ethyl)-amide, A-331440 for 4'-[3-((R)-3-Dimethylamino-pyrrolidin-1-yl)-propoxy]-biphenyl-4-carbonitrile, A-349821 for {4'-[3-((2R,5R)-2,5-Dimethyl-pyrrolidin-1-yl)-propoxy]-biphenyl-4-yl}-morpholin-4-yl-methanone, A-358239 for 4-{2-[2-((R)-2-Methyl-pyrrolidin-1-yl)-ethyl]-benzofuran-5-yl}-benzonitrile and A-431404 for (4-Fluoro-phenyl)-{2-[2-((R)-2-methyl-pyrrolidin-1-yl)-ethyl]-benzofuran-5-yl}-methanone.

Cloning of the histamine H_3 receptor isoforms. The human histamine H_3 receptor gene was cloned using human thalamus poly-A RNA (Clontech, Palo Alto, CA) with RT-PCR methods using primers designed according to the published human histamine H_3 receptor gene sequences (GenBank accession number AF140538; Lovenberg, et al., 2006). The cDNAs for the human full-length ($hH_3R(445)$) and a shorter histamine H_3 receptor isoform ($hH_3R(365)$), with an 80 amino acid deletion from the third intracellular loop, were cloned into the pCIneo expression vector.

Analysis of H_3R isoform expression. Tissue expression of H_3R isoforms was analyzed by RT-PCR. 1 μg of human mRNA (Clontech, Mountain View, CA) were reverse-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and amplified for 35 cycles of 95° C for 45 sec, 60° C for 1.5 min, 72° C for 2 min, and a final extension at

72° C for 7 min using 1 unit of Platinum Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA). The 5' forward and the 3' reverse primers used were F754 (nt 754-778) and R1406 (nt 1383-1406) (Cogé et al., 2001). The PCR products were subcloned into pCR Blunt II-TOPO cloning vector (Invitrogen) and sequenced. PCR products were analyzed using agarose gels stained with ethidium bromide.

Cell culture. The H_3R isoforms were stably expressed in rat C6 glioma cells and were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin in presence of 400 µg/ml G-418.

Radioligand binding. Cells were scraped from their dishes; centrifuged (3 minutes, 1000 rpm) and the pellets were stored at -20°C until use. Before use, the pellets were dissolved in 50 mM Na_2HPO_4, pH 7.4 (for N^{α}-[methyl-^{3}H]histamine and [^{3}H]A-349821) or in 25 mM Tris, 145 mM NaCl and 5 mM $MgCl_2$ (for [^{125}I]-Iodophenpropit) and homogenized for 2 seconds (40 Watt, Labsonic 1510). The cell homogenates (10-20 µg) were incubated for 60 minutes at 25°C with or without competing ligands in a total volume of 200 µl. For competition-binding experiments 0.6 nM N^{α}-[methyl-^{3}H]histamine (83.0 Ci/mmol) or 2.5 nM [^{125}I]-Iodophenpropit was used. Saturation experiments were perform with various concentrations of N^{α}-[methyl-^{3}H]histamine (~0.1 – 20 nM), [^{125}I]-Iodophenpropit (~0.5 – 50 nM), or [^{3}H]A-349821 (~0.01 – 1.5 nM) and non-specific binding was defined by 100 µM thioperamide. The incubation was terminated by rapid filtration over polyethylenimine (0.3 %) pretreated Unifilter GF/C filterplates with two subsequent washes with ice cold 50 mM Tris-HCl (pH 7.4). Radioactivity retained on the filter was determined by liquid scintillation counting on the Microbeta Trilux with 25 µl Microscint "O".

GTPγS binding assay. HEK cell membranes expressing the human H_3R were prepared by homogenization in cold buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM $MgCl_2$, and protease inhibitors. The homogenate was centrifuged two times at 40,000xg for 20 min at 4°C and the resulting pellet was resuspended in buffer containing 50 mM Tris HCl (pH 7.4), 5 mM EDTA, and 10 mM $MgCl_2$. Glycerol and bovine serum albumin (BSA) were added to a final concentration of 10% glycerol and 1% BSA prior to freezing the membranes. The inverse agonist activity of H_3R antagonists was determined as described previously (Krueger et al., 2005). In brief, membranes were diluted in GTPγS assay buffer (25 mM HEPES, 2.5 mM $MgCl_2$, and 75 mM NaCl, pH 7.4) and 10 µg of membrane protein was incubated in a 96-well deep-well block in the presence of 5.0 µM unlabeled GDP, approximately 0.5 nM of [^{35}S]-GTPγS, and various concentrations of H_3R antagonists. Samples were subsequently incubated at 37°C for 20 min. For assays to determine antagonist activity, (*R*)-α-methylhistamine (30 nM) was added in addition to the assay components described above and the samples were incubated at 37°C for 5 min. The assays were terminated by the addition of cold buffer (50 mM Tris-HCl, 75 mM NaCl, and 2.5 mM $MgCl_2$, pH 7.6) and subsequent harvesting by vacuum filtration onto a Packard Unifilter 96-well GF/B plate (Perkin Elmer Life Sciences). After extensive washing, the plates

were dried, Microscint 20 was added to the samples, and the amount of bound [^{35}S]-GTPγS was determined utilizing the Topcount (Perkin Elmer Life Sciences, Boston, MA). The percentage of [^{35}S]-GTPγS bound in each sample was calculated as a percentage of that bound to control samples incubated in the absence of histamine H_3R ligands. Triplicate determinations were obtained at each concentration and the data were analyzed using GraphPad Prism (San Diego, CA) to obtain EC_{50} or IC_{50} values and Hill slopes. pK_b values were calculated using the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973; Leff and Dougall, 1993) The mean ± S.E.M. of at least three independent experiments is reported.

Measurement of cAMP. C6 cells stably expressing the H_3R were washed once with DMEM supplemented with 25 mM HEPES (pH 7.4 at 37°C) and preincubated in the same medium for 30 minutes at 37°C. Thereafter, $5 \cdot 10^3$ cells/50 μl was added per well to a 96-well plate containing the respective ligands in 50 μl DMEM supplemented with 0.3 mM 3-isobutyl-1-methylxanthine and 1 μM forskolin (final concentration 0.5 μM). After 10 minutes incubations were terminated by the addition of 20 μl 0.3 mg/ml saponin to each well to lyse the cells. Subsequently, cAMP levels were determined with a competitive protein kinase A (PKA) binding assay, as described. Protein kinase A-containing fraction was isolated from bovine adrenal glands, as described (Drutel et al., 2001). Briefly, a PKA-containing fraction was isolated from bovine adrenal glands, which were homogenized in 10 volumes of 100 mM Tris-HCl, 250 mM NaCl, 10 mM EDTA, 0.25 M sucrose, and 0.1% 2-mercaptoethanol (pH 7.4 at 4 °C) and centrifuged for 60 min at 30.000 x g at 4°C. The supernatant, containing PKA, was carefully recovered and frozen in 1 ml aliquots at -80 °C. Before use, the PKA was diluted 12-fold in ice-cold phosphate buffer saline (PBS) and kept on ice. To each well 20 μl 0.6 nM [^{3}H]-cAMP in PBS (48.0 Ci/mmol) and 60 μl PKA in PBS was added. After 30 minutes the reaction was terminated by filtration over Unifilter GF/B filterplates with two subsequent washes with 200 μl ice cold 50 mM Tris-HCl (pH 7.4). Retained radioactivity was determined by liquid scintillation counting on the Microbeta Trilux with 50 μl Microscint "O". The amount of cAMP in each sample was calculated with GraphPad Prism version 4.01 for Windows, (GraphPad Software, San Diego, California, USA), using a generated standard cAMP curve (0.1 mM – 10 pM).

Statistical analysis. statistical analyses were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, USA). Differences among means were evaluated by ANOVA, followed by the Dunnett's post test. For all analyses, the null hypothesis was rejected at the 0.05 level.

Chapter 5

G-protein coupling of human histamine H_3 receptor isoforms

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Abstract

In this study, we investigated if different members of the $G\alpha_{i/o}$ family of G-proteins differentially couple to splice variants of the hH_3R that have deletions in the third intracellular loop. To this end, we tested signaling to a cAMP responsive element (CRE)-reporter gene in COS-7 cells co-transfected with PTX insensitive mutant $G\alpha_{i/o}$-proteins and the $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ or $hH_3R(329)$. Our results reveal that all hH_3R isoforms are able to couple to all the tested $G\alpha_{i/o}$-proteins, except for the $hH_3R(329)$ that did not signal via $G\alpha_{i3}$. The agonist-mediated responses via $hH_3R(445)$, $hH_3R(415)$ and $hH_3R(365)$ are more efficacious via $G\alpha_{i2}$ or $G\alpha_{oB}$-proteins, whereas agonists potencies are unaffected. In contrast, the inverse agonist thioperamide is more potent when $G\alpha_{i2}$ or $G\alpha_{oB}$-proteins are co-expressed while the efficacies of the inverse agonists are independent of the co-expressed G-protein. In view of the differential neuronal expression of $G\alpha_{i/o}$ proteins, these data might of physiological relevance and add to our understanding of H_3R pharmacology.

Introduction

The histamine H_3 receptor (H_3R) is predominantly expressed in the central nervous system (CNS) were it acts as a presynaptic autoreceptor that regulates the release and synthesis of histamine, as well as the release of several other neurotransmitters (Leurs et al., 2005; Haas et al., 2008). Based on a large number of preclinical studies, H_3R antagonists are considered to be beneficial in the treatment of obesity, sleep disorders and various cognitive disorders like attention-deficit hyperactivity disorder (ADHD), schizophrenia and Alzheimer's disease (Celanire et al., 2005; Esbenshade et al., 2006).

The H_3R is a G-protein coupled receptor (GPCR) that, via the pertussis toxin (PTX)-sensitive family of $G\alpha_{i/o}$-proteins, mediates various signal transductions pathways (Bongers et al., 2007a). Soon after the cloning of the human H_3R (hH_3R) (Lovenberg et al., 1999) it became apparent that several hH_3R isoforms exist (Hancock et al., 2003; Bongers et al., 2007a). Some of these hH_3R splice variants have deletions in the third intracellular loop (3IL), a region that has been implicated in the specificity of G-protein coupling (Wong, 2003).

From the 20 known hH_3R isoforms, so far only the $hH_3R(445)$, $hH_3R(373)$ and the $hH_3R(365)$ have been shown to bind H_3R radioligands and to show a functional response (Cogé et al., 2001; Wellendorph et al., 2002; Bongers et al., 2007b). Although Cogé et al. initially described the $hH_3R(365)$ as a non-functional GPCR,

Wellendorph et al. showed it to be functional in a R-SAT™ reporter assay, a discrepancy that can likely be ascribed to the high constitutive activity of the $hH_3R(365)$ (Bongers et al., 2007b). The other thus far characterized hH_3R isoforms, the $hH_3R(431)$, $hH_3R(301)$ and $hH_3R(200)$, did not exhibit any H_3R radioligand binding nor any functional responses, likely attributable to the splicing of residues that are structurally important for GPCRs. However, these non-functional hH_3R isoforms might still function in a dominant negative manner as has been shown for the rat H_3R isoforms lacking TM7 (Bakker et al., 2006).
In this study, we characterized the G-protein coupling specificity of the $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ or $hH_3R(329)$. hH_3R isoforms for five members of the $G\alpha_{i/o}$-family of G-proteins, namely $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$ and $G\alpha_{oB}$ (Milligan and Kostenis, 2006), to evaluate if the hH_3R differentially couples to the different $G\alpha_{i/o}$-proteins and if the deletions in the 3IL of the tested hH_3R isoforms affected G-protein coupling. To this end, we expressed the four different hH_3R isoforms with deletions in the 3IL in combination with $G\alpha_{i/o}$-proteins rendered PTX-insensitive by mutagenesis (Milligan, 1988). These PTX-insensitive mutant $G\alpha_{i/o}$-proteins contain a mutated cysteine, that is normally ADP-ribosylated by PTX (West et al., 1985), consequently rendering them insensitive to PTX. Upon transfection, all endogenous $G\alpha_{i/o}$-proteins can be inhibited by PTX-treatment and GPCR signaling can only occur through the co-transfected PTX-insensitive mutant $G\alpha_{i/o}$-proteins.

Results

PTX sensitive hH_3R-mediated CRE activation in transfected COS-7 cells

To demonstrate functional expression of the human H_3 receptor (hH_3R) expressed in COS-7 cells, we co-transfected COS-7 cells with the $hH_3R(445)$ and a cAMP responsive element (CRE) reporter-gene and evaluated the modulation of the forskolin-stimulated CREB (cAMP response element-binding) activation. In this assay, an increase in intracellular cAMP levels leads to an increase in activation of the CRE-binding protein, which results in CRE-mediated transcription of the luciferase reporter gene. COS-7 cells co-transfected with the CRE reporter gene and the $hH_3R(445)$ show a 4-fold increase over the basal CREB-activity upon stimulation with 3 μM forskolin, a direct activator of adenylyl cyclase. Subsequent co-incubation with the H_3R agonists histamine or immepip, leads to a dose-dependent decrease (40%) in forskolin-stimulated CREB-activity (Figure 1A, B). Co-incubation with the H_3R inverse agonist thioperamide results in a 50% increase in forskolin-stimulated CREB-activity (Figure 1B), indicating constitutive activity of the $hH_3R(445)$ in this experimental model. The potencies found in the CRE reporter gene assay in COS-7 cells for the tested H_3R ligands histamine (pEC_{50}= 7.7 $\pm$ 0.2),

immepip (pEC_{50}= 8.5 ± 0.4) and thioperamide (pEC_{50}= 6.7 ± 0.3) correspond well with their earlier reported potencies found in a guanosine-5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding and cAMP assay (Bongers et al., 2007b).

Pre-treatment of transfected COS-7 cells for 24 hours with 100 ng/ml PTX completely abolishes the hH_3R(445)-mediated modulation of the CRE reporter gene and reveals an increase in basal CREB-activity, again indicating constitutive signaling of the hH_3R(445) to CREB via PTX-sensitive $G\alpha_{i/o}$-proteins in these transiently transfected COS-7 cells (Figure 1B). Moreover, PTX-treatment increases the CREB-activity to the same levels as the maximum response of the inverse agonist thioperamide and no additional increase is seen upon stimulation with the inverse agonist thioperamide.

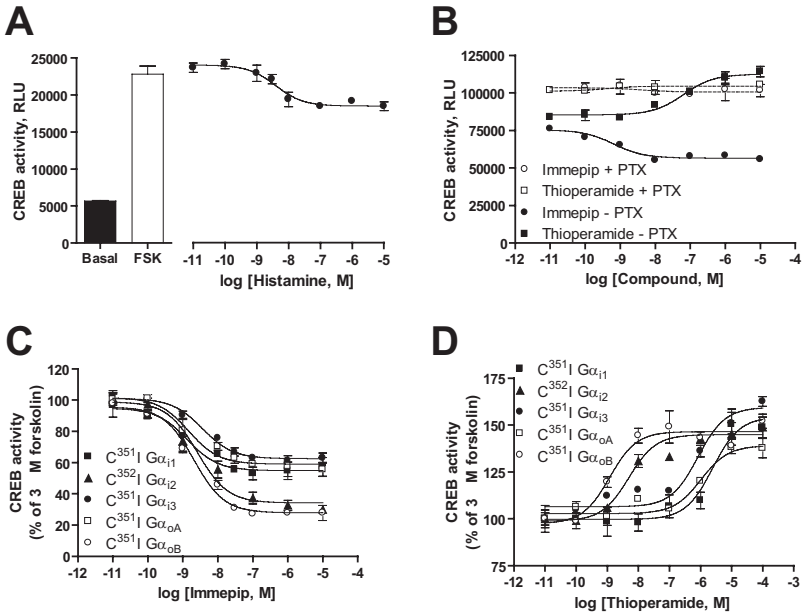

Figure 1. Functional responses of COS-7 cells transiently expressing the hH_3R(445) and the CRE-reporter gene. (A) Stimulation with 3 μM forskolin results in a 4-fold increase of basal signal and subsequent co-incubation with the hH_3R agonist histamine, results in a dose-dependent decrease of the forskolin-induced CRE response. (B) PTX (100 ng/ml, o/n) treatment completely abolishes the response to the hH_3R specific agonist immepip and the hH_3R inverse agonist thioperamide. (C and D) Additional co-expression of PTX-insensitive mutant $G\alpha_{i1}(C^{351}I)$, $G\alpha_{i2}(C^{352}I)$, $G\alpha_{i3}(C^{351}I)$, $G\alpha_{oA}(C^{351}I)$, $G\alpha_{oB}(C^{351}I)$ -proteins rescues the dose-dependent hH_3R-mediated modulation of the CRE-reporter gene. Results represent the mean ± S.E.M of a typical experiment.

Co-transfection of PTX-insensitive mutant $G\alpha_{i/o}$-proteins reveals a differential rescue of functional $hH_3R(445)$ response after PTX treatment

In order to investigate the role of the individual $G\alpha_{i/o}$-proteins in the hH_3R-mediated responses, COS-7 cells were pre-treated with 100 ng/ml PTX and transfected with the CRE reporter gene, the $hH_3R(445)$ and PTX-insensitive mutant $G\alpha_{i/o}$-proteins. Subsequent stimulation with the H_3R agonist immepip or the H_3R inverse agonist thioperamide shows that co-expression of PTX-insensitive mutant $G\alpha_{i/o}$-proteins rescues the $hH_3R(445)$ mediated CREB responses in PTX treated COS-7 cells (Figure 1C, D and 5). For the H_3R agonist immepip a significantly more pronounced inhibition of the CREB-activation was found after co-expression of either $C^{352}I\ G\alpha_{i2}$ or $C^{351}I\ G\alpha_{oB}$ (63±4 and 63±3% inhibition, respectively) compared to the co-expression with the $C^{351}I\ G\alpha_{i1}$, $C^{351}I\ G\alpha_{i3}$ or $C^{351}I\ G\alpha_{oA}$ subunits (49±4, 45±3 and 46±2% inhibition, respectively). At the same time, no significant differences in pEC_{50} values for immepip were found after co-expression of $hH_3R(445)$ with the different G-protein subunits (Figure 1C and Figure 5). In contrast, for the H_3R inverse agonist thioperamide significant lower potencies (pEC_{50} = 5.1±0.2, 5.8±0.1 and 5.2±0.3, respectively) were found after co-expression of the $hH_3R(445)$ with $C^{351}I\ G\alpha_{i1}$, $C^{351}I\ G\alpha_{i3}$ or $C^{351}I\ G\alpha_{oA}$ compared to co-expression with $C^{351}I\ G\alpha_{i2}$ or $C^{351}I\ G\alpha_{oB}$ (pEC_{50} = 8.4±0.1 and 7.4±0.2, respectively). No significant differences in inverse agonistic efficacies of thioperamide was found upon co-expression of the $hH_3R(445)$ with various PTX-insensitive mutant $G\alpha_{i/o}$-proteins (Figure 1D).

No differences in expression of the PTX-insensitive mutant $G\alpha_{i/o}$-proteins or the hH_3R

To test if the PTX-insensitive mutant $G\alpha_{i/o}$-proteins were expressed at a similar level we performed Western blot analysis with a generic $G\alpha_{i/o/t/z}$-protein antibody on cell lysates of COS-7 cells co-expressing the $hH_3R(445)$ and one of the PTX-insensitive mutant $G\alpha_{i/o}$-proteins. A significant over-expression (5-fold) compared to mock transfected cells was found for all PTX-insensitive mutant $G\alpha_{i/o}$-proteins, but no significant differences were found between the expression levels of the individual PTX-insensitive mutant $G\alpha_{i/o}$-proteins (Figure 2A).

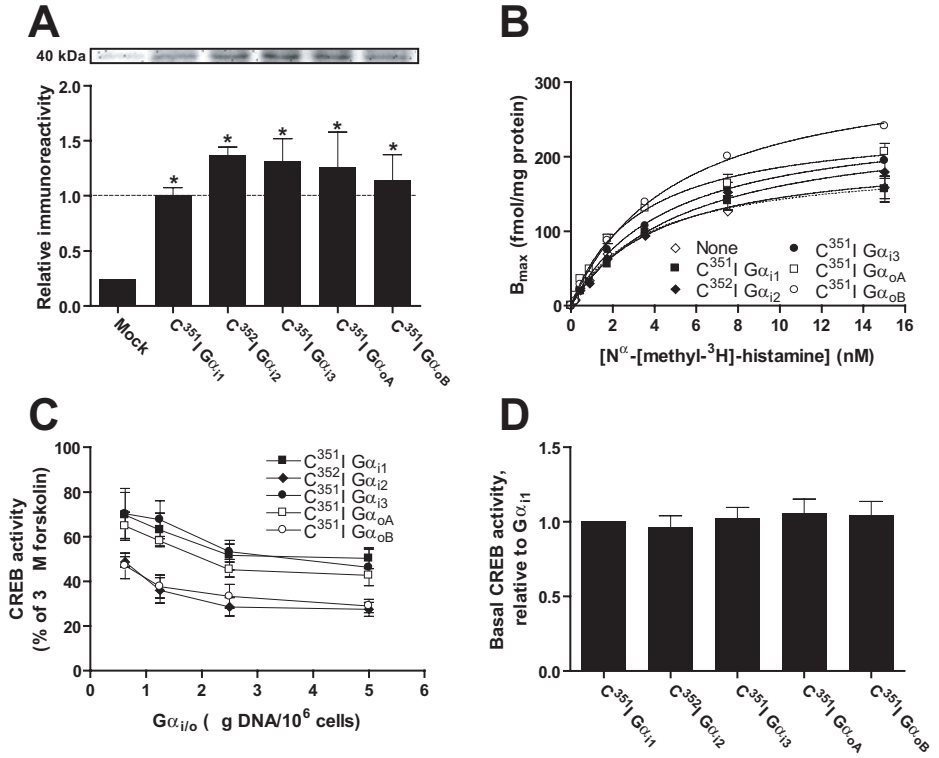

Figure 2. Characterization of COS-7 cells transiently expressing the $hH_3R(445)$ and PTX-insensitive mutant $G\alpha_{i/o}$-proteins. (A) Relative immunoreactivity of the PTX-insensitive mutant $G\alpha_{i/o}$-proteins to the $G\alpha_{i/o/t/z}$ specific antibody. * $p<0.05$ *versus* mock. (B) Maximal specific binding (B_{max}) of N^{α}-[methyl-^{3}H]-histamine on hH_3R expressing membranes co-transfected with or without the PTX-insensitive mutant $G\alpha_{i/o}$-proteins. (C) Relative inhibition of the CRE-reporter gene after stimulation with the hH_3R agonist immepip with varying amounts of the PTX-insensitive mutant $G\alpha_{i/o}$-proteins. (D) Relative basal inhibition of the CRE-reporter gene upon co-expression of a PTX-insensitive mutant $G\alpha_{i/o}$-protein and the $hH_3R(445)$. Results represent the mean ± S.E.M. of three independent experiments and a representative blot (A) or the means ± S.E.M of a typical experiment (B-D). * $p<0.05$ *versus* mock.

To examine if expression of the different PTX-insensitive mutant $G\alpha_{i/o}$-proteins affected $hH_3R(445)$ expression levels, we also determined the maximal specific binding (B_{max}) of N^{α}-[methyl-^{3}H]-histamine on membranes of COS-7 cells transiently co-transfected with the $hH_3R(445)$ and the various PTX-insensitive mutant $G\alpha_{i/o}$-proteins. Saturation binding analysis revealed a B_{max} of 117 ± 28 fmol/mg protein and a best fit for a single binding site with a K_d value of 3.4 ± 0.4 in the absence of PTX-insensitive mutant $G\alpha_{i/o}$-proteins (Table 1). Expression of the

various PTX-insensitive mutant $G\alpha_{i/o}$-proteins did not significantly affect the B_{max} of the $hH_3R(445)$ as determined with N^{α}-[methyl-^{3}H]-histamine (Table 1). Moreover, no significant differences were found for the B_{max} or K_d of N^{α}-[methyl-^{3}H]-histamine upon co-expression of $hH_3R(445)$ and the various PTX-insensitive mutant $G\alpha_{i/o}$-proteins as determined with saturation binding analysis (Figure 2B and Table 1).
To investigate how $G\alpha_{i/o}$-protein expression affects the maximal percentage of inhibition of the $hH_3R(445)$-mediated CREB response, we co-transfected the PTX-insensitive mutant $G\alpha_{i/o}$-proteins in increasing amounts. For all the tested amounts of DNA of the various PTX-insensitive mutant G-proteins co-expressed with $hH_3R(445)$, $C^{352}I$ $G\alpha_{i2}$ and $C^{351}I$ $G\alpha_{oB}$ showed a more pronounced inhibition of the CRE-mediated response by the H_3R agonist immepip compared to $C^{351}I$ $G\alpha_{i1}$, $C^{351}I$ $G\alpha_{i3}$ and $C^{351}I$ $G\alpha_{oA}$ (Figure 2C). Furthermore, amounts of DNA concentration above 2.5 $\mu g/10^6$ cells did not lead to a further decrease in the immepip-induced $hH_3R(445)$ mediated CREB response (Figure 2C), indicating that G-protein expression is not limiting at DNA amounts above 2.5 $\mu g/10^6$ cells. Furthermore, the basal CREB-activity of the $hH_3R(445)$ was not different after co-expression with the various PTX-insensitive mutant $G\alpha_{i/o}$-proteins (Figure 2D).

Table 1. Maximal specific binding (B_{max}) and equilibrium dissociation constant (K_d) of N^{α}-[methyl-^{3}H]-histamine in PTX-treated membranes of COS-7 cells transiently co-expressing the $hH_3R(445)$ and a PTX-insensitive mutant $G\alpha_{i/o}$-protein. Values are expressed as mean ± S.E.M.

PTX-insensitive mutant $G\alpha_{i/o}$-proteins	B_{max} (fmol/mg protein)	K_d (nM)	n
$C^{351}I$ $G\alpha_{i1}$	138 ± 51	2.6 ± 0.5	5
$C^{352}I$ $G\alpha_{i2}$	139 ± 42	3.7 ± 0.5	5
$C^{351}I$ $G\alpha_{i3}$	159 ± 51	3.3 ± 0.5	5
$C^{351}I$ $G\alpha_{oA}$	146 ± 37	2.5 ± 0.5	5
$C^{351}I$ $G\alpha_{oB}$	148 ± 52	2.6 ± 0.4	5

Does alternative splicing in intracellular loop 3 affect G-protein coupling?
Alternative splicing of the hH_3R gene leads to the generation of several naturally occurring hH_3R isoforms (Bongers et al., 2007a). Among these are hH_3R isoforms with large deletions in the intracellular loop 3 (IL3) (Figure 3). Expression of four these hH_3R isoforms, the $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ and $hH_3R(329)$, in COS-7 cells that were co-transfected with a CRE reporter gene allowed for the functional characterization of these hH_3R isoforms (Figure 4). Stimulation with the hH_3R agonists immepip resulted in a dose-dependent decrease of the forskolin induced CREB-activity. The percentage inhibition was most pronounced with the $hH_3R(415)$, followed by the $hH_3R(445)$. The $hH_3R(365)$ and $hH_3R(329)$ were found

to be the least efficacious in coupling to the G-proteins endogenously present in COS-7 cells. All tested hH_3R isoforms respond equally potent to the H_3R-agonist. In contrast, the H_3R inverse agonist thioperamide is significantly less potent and more efficacious with the $hH_3R(365)$, a phenomenon that is most likely explained by an increase in constitutive activity of this hH_3R isoforms, as described earlier (Bongers et al., 2007b).

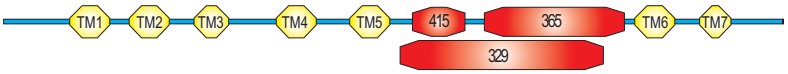

Figure 3. Schematic representation of the hH_3R isoforms. Indicated in red is the region that is lacking due to alternative splicing in the respective isoform.

To test if alternative splicing of the hH_3R mRNA affects $G\alpha_{i/o}$-protein coupling, we tested the $hH_3R(415)$, $hH_3R(365)$ and $hH_3R(329)$ isoforms in the CRE reporter-gene assay using PTX-treated COS-7 cells co-transfected with the various PTX-insensitive mutant $G\alpha_{i/o}$-proteins. Upon co-expression in COS-7 cells the expression levels of the various hH_3R isoforms were similar. Neither the B_{max} nor the K_d values of N^{α}-[methyl-^{3}H]-histamine were significantly different among the tested hH_3R isoforms (Table 2).

Table 2. Maximal specific binding (B_{max}) and equilibrium dissociation constant (K_d) of N^{α}-[methyl-^{3}H]-histamine in membranes of COS-7 cells transiently expressing hH_3R isoforms. Values are expressed as mean ± S.E.M.

hH_3R isoform	B_{max} (fmol/mg protein)	K_d (nM)	n
445	117 ± 28	3.4 ± 0.4	7
415	179 ± 60	3.0 ± 0.7	3
365	131 ± 18	1.8 ± 0.5	6
329	80 ± 26	1.9 ± 0.4	4

To test for functional differences between the various isoforms, we investigated the modulation of the CRE-mediated luciferase after co-expression of PTX-insensitive mutant $G\alpha_{i/o}$-proteins by the various hH_3R isoforms (Figure 5, 6). For the $hH_3R(415)$, we found similar results as obtained for the $hH_3R(445)$. The maximal inhibition by immepip was substantially higher after co-expression of $hH_3R(415)$ with $C^{352}I\ G\alpha_{i2}$ and $C^{351}I\ G\alpha_{oB}$ (67±1 and 66±6% inhibition, respectively) compared to co-expression with the $C^{351}I\ G\alpha_{i1}$, $C^{351}I\ G\alpha_{i3}$ and $C^{351}I\ G\alpha_{oA}$ subunits (46±8, 39±3 and 50±1 % inhibition, respectively). No significant differences in potencies for immepip were observed (Figure 5A, 6). At the same time, the effects of the inverse agonist thioperamide were significantly more potent upon co-expression of $hH_3R(415)$ with $C^{352}I\ G\alpha_{i2}$ and $C^{351}I\ G\alpha_{oB}$ (Figure 5B, 6). For the $hH_3R(365)$ we

observed an increase in efficacy for the inverse agonists and conversely a decrease in agonist efficacy, indicating constitutive activity, as was reported previously (Bongers et al., 2007b). In contrast to the other tested hH_3R isoforms, we found no differences in potency for the inverse agonist thioperamide upon co-expression of the various PTX-insensitive mutant $G\alpha_{i/o}$-proteins (Figure 5C-D, 6). For the $hH_3R(329)$ isoform we observed an overall decrease in efficacy for immepip; no inhibition of CRE-mediated transcription could be measured upon co-expression of this isoform with the $C^{351}I$ $G\alpha_{i3}$ subunit (Figure 5E, 6). For the inverse agonist thioperamide a potency shift was observed upon co-expression of $C^{351}I$ $G\alpha_{i1}$, $C^{351}I$ $G\alpha_{i3}$ or $C^{351}I$ $G\alpha_{oA}$, similar to $hH_3R(445)$ and $hH_3R(415)$ (Figure 5F, 6).

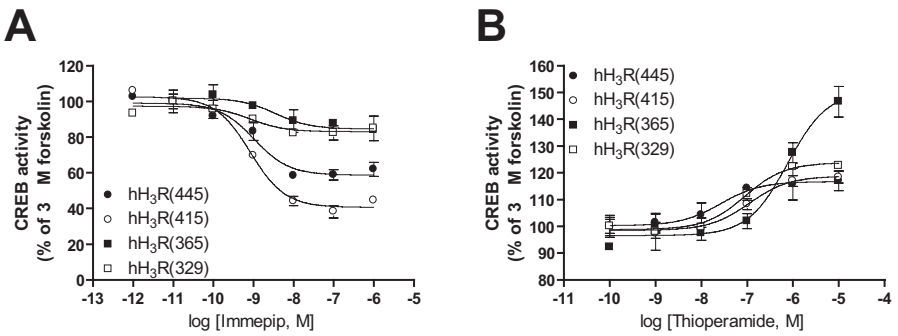

Figure 4. Functional responses of with endogenous G-proteins in COS-7 cells transiently co-expressing the CRE-reporter gene $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ or $hH_3R(329)$ isoforms. Cells were stimulated with the hH_3R agonists immepip (A) or the hH_3R inverse agonist thioperamide (B). Results represent the mean ± S.E.M of a typical experiment.

Finally, we also determined the equilibrium dissociation constant (pK_i) of thioperamide in a competitive binding study with N^{α}-[methyl-^{3}H]-histamine in membranes of COS-7 cells transiently expressing hH_3R isoforms and PTX-insensitive mutant $G\alpha_{i/o}$-proteins. The binding curves are best fit by a one-site binding and the obtained pK_i values are not significantly different between de various PTX-insensitive mutant $G\alpha_{i/o}$-proteins (Table 3).

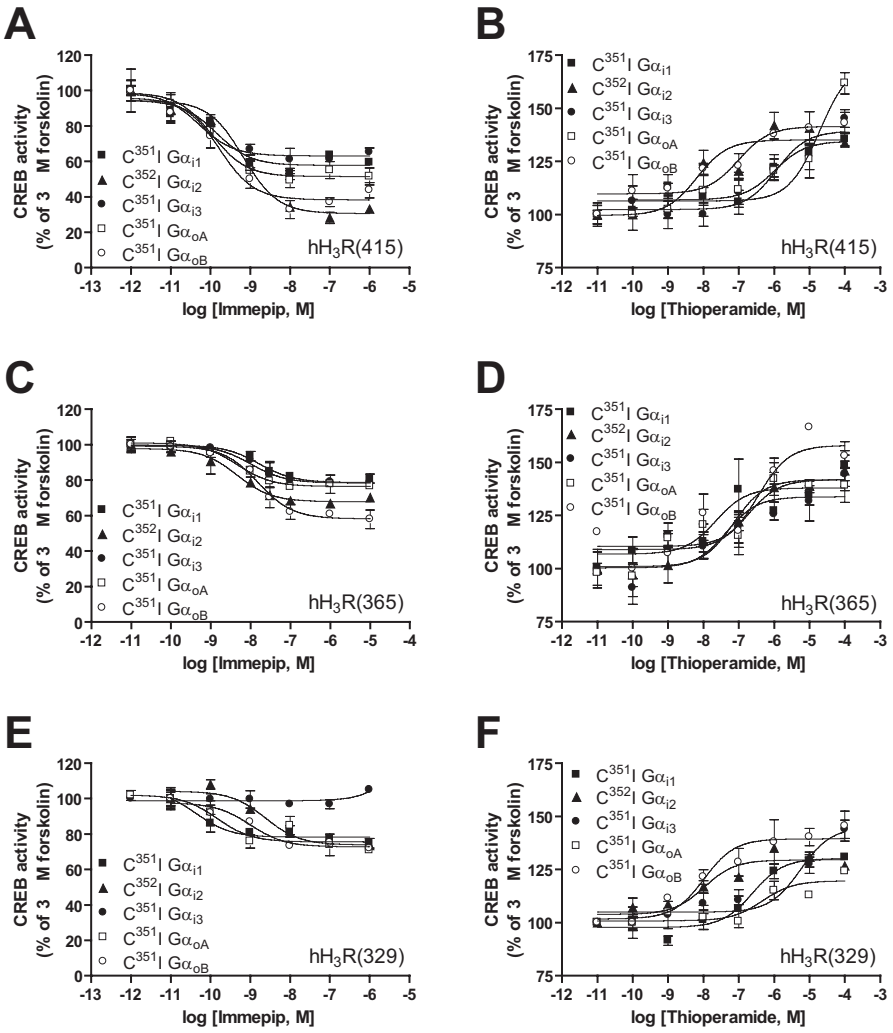

Figure 5. Functional responses of PTX-treated COS-7 cells transiently co-expressing the CRE-reporter gene, PTX-insensitive mutant $G\alpha_{i/o}$-proteins and $hH_3R(415)$ (A, B) or $hH_3R(365)$ (C, D) or $hH_3R(329)$ (E, F) isoforms. Cells were stimulated with the hH_3R agonists immepip or the hH_3R inverse agonist thioperamide. Results represent the mean ± S.E.M of a typical experiment.

Table 3. Equilibrium dissociation constant (pK_i) of thioperamide as determined in a competitive binding study with N^{α}-[methyl-^{3}H]-histamine in membranes of COS-7 cells transiently co-expressing a hH_3R isoform and a PTX-insensitive mutant $G\alpha_{i/o}$-protein. Values are expressed as mean ± S.E.M.

PTX-insensitive mutant $G\alpha_{i/o}$-proteins	hH_3R isoform 445	415	365	329
$C^{351}I\ G\alpha_{i1}$	6.9 ± 0.1	6.9 ± 0.3	6.6 ± 0.1	6.7 ± 0.1
$C^{352}I\ G\alpha_{i2}$	7.0 ± 0.1	7.0 ± 0.2	6.6 ± 0.2	7.0 ± 0.1
$C^{351}I\ G\alpha_{i3}$	6.9 ± 0.1	6.5 ± 0.2	6.6 ± 0.1	6.9 ± 0.1
$C^{351}I\ G\alpha_{oA}$	6.7 ± 0.1	6.5 ± 0.2	6.4 ± 0.3	6.8 ± 0.1
$C^{351}I\ G\alpha_{oB}$	6.8 ± 0.1	6.8 ± 0.2	6.6 ± 0.1	6.8 ± 0.1

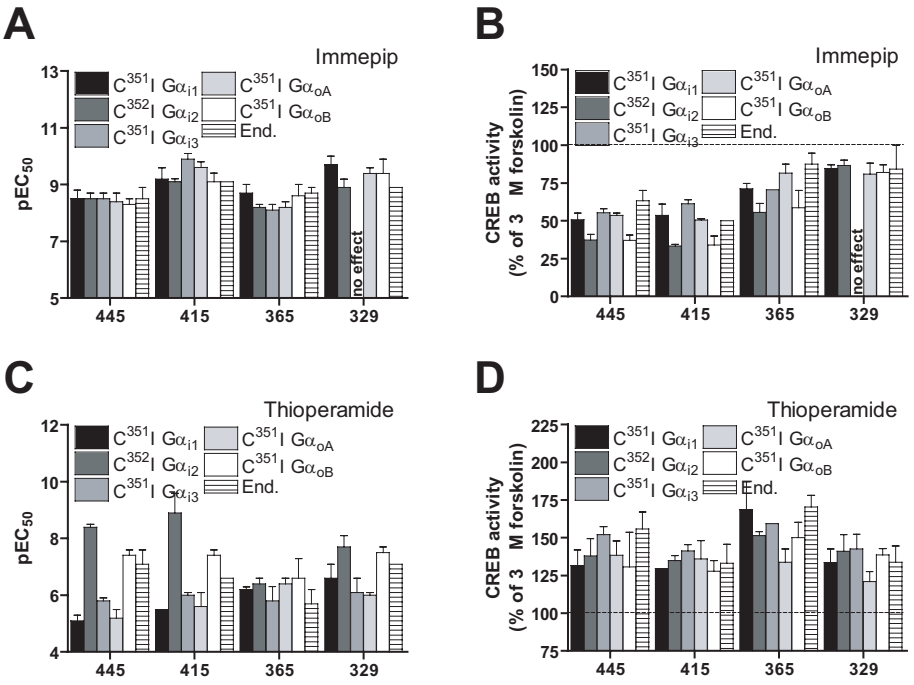

Figure 6. Graphical representation of functional responses obtained in PTX-treated COS-7 cells transiently expressing the CRE-reporter gene, PTX-insensitive mutant $G\alpha_{i/o}$-proteins and the $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ or $hH_3R(329)$. For comparison, CRE-activity obtained with endogenous G-proteins present in COS-7 cells transiently expressing the CRE-reporter gene and the $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ or $hH_3R(329)$ are shown as well (End.) Results represent the mean ± S.E.M of 3-10 experiments.

Discussion

The cloning of the hH_3R (Lovenberg et al., 1999) subsequently led to the discovery of various hH_3R isoforms. However, the potential roles of the pharmacological differences between these isoforms remain elusive (Hancock et al., 2003; Bongers et al., 2007a). In some of the hH_3R isoforms deletions in the 3IL occur via alternative splicing of the hH_3R mRNA. The 3IL is suggested to interact with various intracellular proteins (Abramow-Newerly et al., 2006), among which are the G-proteins (Wong, 2003). Already before the cloning of the hH_3R receptor it was suggested that the H_3R was a $G\alpha_{i/o}$-protein coupled receptor as shown by the sensitivity to PTX of H_3R-mediated responses in various assays systems (Clark et al., 1993; Endou et al., 1994; Clark and Hill, 1995). This was confirmed upon expression of the cloned hH_3R receptor in SK-N-MC cells by showing that the hH_3R-mediated inhibition of the forskolin-induced cAMP formation was sensitive to PTX (Lovenberg et al., 1999). *In vivo* H_3R-mediated inhibition of cAMP levels was shown to be important for the H_3R-mediated synthesis of histamine and the H_3R-mediated exocytosis of norepinephrine (Gomez-Ramirez et al., 2002; Seyedi et al., 2005; Torrent et al., 2005; Moreno-Delgado et al., 2006).

The $G\alpha_{i2}$- and $G\alpha_{i3}$-proteins are widely expressed, whereas expression of the $G\alpha_{i1}$-, $G\alpha_{oA}$- and $G\alpha_{oB}$-proteins is more restricted to the CNS (Milligan and Kostenis, 2006). Especially the $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ have been described to inhibit the various subtypes of adenylyl cyclase. Due to the relative high expression of the $G\alpha_{i/o}$-proteins, activation of the $G\alpha_{i/o}$-proteins may lead to the release of high amounts of $\beta\gamma$-subunits. Consequently, activation of $G\alpha_{i/o}$-coupled GPCRs is considered to be the major source of free $\beta\gamma$-subunits and the subsequent activation $\beta\gamma$-mediated processes. Moreover, it is suggested that for $G\alpha_{oA}$ and its splice variant $G\alpha_{oB}$, the release of $\beta\gamma$-subunits is primary mode of signal transduction (Wettschureck and Offermanns, 2005).

In this study we tried to evaluate if the hH_3R preferentially couples to any of the five tested members of the $G\alpha_{i/o}$-family of G-proteins and.if the deletions in the 3IL of three hH_3R isoforms affects G-protein coupling. We show that the four tested hH_3R isoforms are able to couple to all tested PTX-insensitive mutant $G\alpha_{i/o}$-proteins, except for the $hH_3R(329)$ for which we did not find a functional response when the PTX-insensitive mutant $G\alpha_{i3}$-protein was co-expressed. For the other tested PTX-insensitive mutant $G\alpha_{i/o}$-proteins we observed a functional response with $hH_3R(329)$, but the inhibition in response to the H_3R agonists immepip obtained with the $hH_3R(329)$ is lower compared to the other hH_3R isoforms. This lower response obtained with the $hH_3R(329)$ cannot be attributed to an increase in constitutive activity as was shown to be the case for the $hH_3R(365)$ (Bongers et al.,

2007b). Additionally, we show that the $hH_3R(445)$, $hH_3R(415)$ and $hH_3R(365)$ all show more pronounced inhibition of the forskolin induced CREB-activity when either the PTX-insensitive mutant $G\alpha_{i2}$ or $G\alpha_{oB}$-proteins were co-expressed compared to the PTX-insensitive mutant $G\alpha_{i1}$, $G\alpha_{i3}$ and $G\alpha_{oA}$-proteins.
For the H_3R agonist immepip the co-expression of different PTX-insensitive mutant $G\alpha_{i/o}$-proteins does not affect potencies. In contrast, for the hH_3R inverse agonist thioperamide coupling of the H_3R with $G\alpha_{i2}$ and $G\alpha_{oB}$ proteins, results in potency values that are comparable to the values obtained with the endogenously present $G\alpha_{i/o}$-proteins, whereas potencies obtained with the other $G\alpha_{i/o}$-proteins are magnitudes lower. Conversely, the binding affinities obtained in a heterologous competitive binding assay with N^{α}-[methyl-^{3}H]-histamine were unaffected by co-expressed of the tested PTX-insensitive mutant $G\alpha_{i/o}$-proteins. The observation that thioperamide is much more potent when PTX-insensitive mutant $G\alpha_{i2}$ and $G\alpha_{oB}$ are co-expression suggests that the action of H_3R inverse agonists is mainly conferred through these two $G\alpha_{i/o}$-proteins. This can not be simply accounted for by an increased affinity of the hH_3R for $G\alpha_{i2}$ and $G\alpha_{oB}$, because one would expect an increase in constitutive activity when $G\alpha_{i2}$ and $G\alpha_{oB}$ are co-expressed compared to the other tested G-proteins, which we did not observe. Currently there is no clear reason why the five tested $G\alpha_{i/o}$-proteins separate in two groups based on the H_3R pharmacology. Sequence alignment of the $G\alpha_{oA}$ and $G\alpha_{oB}$ suggest that the pharmacological difference that we observe for these G-proteins in H_3R coupling is located in the C-terminal domain of the G-proteins, because up to residue 249 the two $G\alpha_{o}$-protein splice variant are identical. Sequence alignment of all five tested $G\alpha_{i/o}$-proteins suggests a region between the α4-helix en β5-sheet, the so called αG-region, to be important as this distinguishes $G\alpha_{i2}$ and $G\alpha_{oB}$ from $G\alpha_{i1}$, $G\alpha_{i3}$ and $G\alpha_{oA}$.. This region is part of the helical domain of the G-protein and is not thought to be part of the GPCR G-protein contact regions (Oldham and Hamm, 2008). However, also regions distant from interface between the GPCR and G-protein are known to affect coupling (Heydorn et al., 2004; Kostenis et al., 2005). Furthermore, the helical domain of the G-protein is implicated in binding to proteins that might indirectly influence GPCR signaling like RGS- and certain effector-proteins (Liu and Northup, 1998; Skiba et al., 1999). Interestingly some RGS proteins are known to discriminate between the different G-proteins. The GDI domain of RGS14 seems only to affect $G\alpha_{i1}$, $G\alpha_{i3}$ and $G\alpha_{oA}$, but not $G\alpha_{i2}$ (Mittal and Linder, 2004). The fact that the differential H_3R coupling only affects H_3R inverse agonists, but not agonist potencies nor constitutive activity, suggests that the $G\alpha_{i/o}$-proteins have a different affinity for the different receptor states and an

enhanced affinity is observed for the G-proteins associated with the active receptor bound to an inverse agonist in case of $G\alpha_{i2}$ and $G\alpha_{oB}$.
In contrast to the functional data, no effects were observed for the N^{α}-[methyl-^{3}H]-histamine competition binding data with thioperamide. This apparent discrepancy between the potency and affinity of thioperamide might be explained by the fact that in our data only one-site binding is observed. In two-site binding curves only the high affinity site is usually affected by G-proteins. Moreover, affinity and potency are not always directly correlated (Mackay, 1990; Kenakin and Onaran, 2002).
The receptor G-protein stoichiometry is known to affect the pharmacology of a receptor (Newman-Tancredi et al., 1997), but only to a certain extent does increasing G-protein levels affect pharmacology (Gazi et al., 2003). This can be explained, since not just the GPCR and the G-protein determine the final effect, as RGS proteins are known to play an important role as well (Hepler, 1999; Jean-Baptiste et al., 2006).
The observed dependence of H_3R pharmacology on G protein co-expression is of great interest, since $G\alpha_o$-proteins are highly expressed in brain and might influence, depending on their relative expression, the H_3R pharmacology in certain brain areas. Moreover, since different H_3R inverse agonists behave differently in certain *in vivo* models (Hancock, 2006), one might speculate that differential H_3R;G-protein combinations are implicated, and that these combinations are potentially brain-region dependent.

Methods and methods

Materials. Dulbecco's Modified Eagle Medium, trypsin-EDTA, penicillin, L-glutamine and streptomycin, bovine serum albumin (BSA) , chloroquine diphosphate, DEAE-dextran, and the TOPO cloning kit were from Invitrogen (Breda, The Netherlands) and fetal calf serum was from Integro (Zaandam, The Netherlands). Culture dishes were from Greiner Bio-one (Wemmel, Belgium). Oligonucleotides were purchased from Isogen Biocience (Maarsen, The Netherlands). Pertussis toxin (PTX) was obtained from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). PAC clones were obtained from the BACPAC Resources Center at the Children's hospital Oakland (Oakland CA, USA). All H_3R ligands were (re-)synthesized at the Vrije Universiteit Amsterdam or at Abbott Laboratories. The enhanced-chemiluminescence assay "Western Lightning" and N^{α}-[methyl-^{3}H]histamine (83 Ci/mmol) were from PerkinElmer Life Sciences (Zaventem, Belgium). The following compound-abbreviations were used: A-349821 for {4'-[3-((2R,5R)-2,5-Dimethyl-pyrrolidin-1-yl)-propoxy]-biphenyl-4-yl}-morpholin-4-yl-methanone. The pTATAlucNEO/CRE121-3 (pTLNC121-3) cAMP response element (CRE) luciferase reporter gene was a kind gift from dr. W. Born (Fluhmann et al., 1998). The PTX-insensitive mutant human $G\alpha_{i/o}$-proteins and the EE-tagged human $G\alpha_{i/o}$-proteins (pcDNA3.1/$G\alpha_{i1}$(C^{351}I), pcDNA3.1/$G\alpha_{i2}$(C^{352}I),

pcDNA3.1/$G\alpha_{i3}(C^{351}I)$, pcDNA3.1/$G\alpha_{oA}(C^{351}I)$, pcDNA3.1/$G\alpha_{oB}(C^{351}I)$, pcDNA3.1/$G\alpha_{i1}$-EE-tagged, pcDNA3.1/$G\alpha_{i2}$-EE-tagged, pcDNA3.1/$G\alpha_{i3}$-EE-tagged, pcDNA3.1/$G\alpha_{oA}$-EE-tagged, pcDNA3.1/$G\alpha_{oB}$-EE-tagged) were obtained from the UMR cDNA Resource Center (Miner Circle Rolla, MO). Antibodies recognizing $G\alpha_{i/o/t/z}$ (D-15) were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Rabbit anti-goat horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA).

Cloning of the histamine H_3 receptor isoforms. The human histamine H_3 receptor (hH_3R) was cloned from the human male PAC clone RP5-1005F21 with the following primers for the three exons of the hH_3R: forward primer GCCATGGAGCGCGCGCCGCC and reverse primer CGACGAGGAAGTCGGA were used for exon 1, forward primer TCCGACTTCCTCGTCGGCGCCTTCTGCATCCC and reverse primer CGCTCGGGTGACCGAC were used for exon 2 and forward primer GTCGGTCACCCGAGCGGTCTCATACCGGGCCC and reverse primer ATGGAGCGCGCGCCGCCCGA were used for exon 3. Subsequently, exon 1 and exon 2 were joined by PCR with forward primer AAGGTACCGCCACCATGGAGCGCGCGCCGCCCG and reverse primer CGCTCGGGTGACCGAC and exon 2 and exon 3 were joined by PCR with forward primer TCCGACTTCCTCGTCGGCGCCTTCTGCATCCC and reverse primer AATCTAGATATCTCACTTCCAGCAGTGCTCC. These two fragments were joined by PCR with forward primer AAGGTACCGCCACCATGGAGCGCGCGCCGCCCG and reverse primer AATCTAGATATCTCACTTCCAGCAGTGCTCC. The subsequent fragment was cloned into pcDNA3.1/V5-HisTOPO vector by TOPO TA cloning (Invitrogen, Breda, The Netherlands) and subsequently subcloned into the pCIneo expression vector. The cloned hH_3R was confirmed to be identical to the published $hH_3R(445)$ sequence (GenBank accession number NM_007232).The $hH_3R(415)$, $hH_3R(365)$ and $hH_3R(329)$ were a kind gift from T.A. Esbenshade. (Neuroscience Research, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, Illinois, USA).

Cell culture and transfection. The H_3R isoforms were transiently expressed in COS-7 cells and were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin. COS-7 cells were transiently transfected using the DEAE-dextran method. The total amount of DNA transfected was maintained constant by addition of pcDNA3.

Reporter-Gene Assay. Cells transiently co-transfected with pTATAlucNEO/CRE121-3 (pTLNC121-3) CRE-luciferase reporter gene (Fluhmann et al., 1998) (10 $\mu g/1 \cdot 10^6$ cells), pCIneo/hH_3R isoforms (2.5 $\mu g/1 \cdot 10^6$ cells) and PTX-insensitive mutant pcDNA3.1/$G\alpha_{i/o}$-proteins (2.5 $\mu g/1 \cdot 10^6$ cells) were seeded in 96-well white plates (Greiner Bio-one, Wemmel, Belgium) in serum-free culture medium. After 48 hours, cells were stimulated and assayed for luminescence by aspiration of the medium and the addition of 25 µL/well luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM

Tris, pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 μM dithiothreitol). After 5 min, luminescence was measured for 1 sec./well on a Victor^2 (PerkinElmer Wallac, Gaithersburg, MD).

Radioligand binding. Cells were scraped from their dishes; centrifuged (3 minutes, 1000 rpm) and the pellets were stored at -20°C until use. Before use, the pellets were dissolved in 50 mM Tris, pH 7.4 (for N^{α}-[methyl-^{3}H]histamine) and homogenized for 2 seconds (40 Watt, Labsonic 1510). The cell homogenates (10-20 μg) were incubated for 60 minutes at 25°C with or without competing ligands in a total volume of 200 μl. For competition-binding experiments 0.6 nM N^{α}-[methyl-^{3}H]histamine (83.0 Ci/mmol). Saturation experiments were perform with various concentrations of N^{α}-[methyl-^{3}H]histamine (~0.1 – 20 nM) and non-specific binding was defined by 100 μM thioperamide. The incubation was terminated by rapid filtration over polyethylenimine (0.3 %) pretreated Unifilter GF/C filterplates with two subsequent washes with ice cold 50 mM Tris-HCl (pH 7.4). Radioactivity retained on the filter was determined by liquid scintillation counting on the Microbeta Trilux with 25 μl Microscint "O".

Western blot analysis. Transiently transfected COS-7 cells were seeded in 6-well plates ($5 \cdot 10^5$ cells/well) in culture medium. After 48 hours the cells were washed with with PBS (pH 7.4 at 4°C). and subsequently lysed in 100 μl radioimmunoprecipitation assay (RIPA) buffer (PBS containing 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 2 μg/ml aprotinin and 2 μg/ml leupeptin), sonicated, separated by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. The membrane was blocked for 60 minutes at RT in T-TBS (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 8.0) buffer containing 5% (v/w) milk powder. Subsequently, the antibody recognizing $G\alpha_{i/o/t/z}$ (D-15) (Santa Cruz Biotechnology, Inc.) (1:500) was incubated overnight at 4°C in T-TBS containing 5% (v/w) BSA and used in combination with a rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (1:5,000), incubated for 60 minutes at RT in T-TBS containing 5% (v/w) milk powder. Immunoreactivity was detected by an enhanced-chemiluminescence assay and directly quantified with a Kodak Image station (PerkinElmer Life and Analytical Sciences, Inc. Boston, MA USA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, USA). Differences among means were evaluated by ANOVA, followed by the Dunnett's post test. For all analyses, the null hypothesis was rejected at the 0.05 level.

Chapter 6

The Akt/GSK-3β axis as a new signaling pathway of the histamine H_3 receptor

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Abstract

Drugs targeting the histamine H_3 receptor (H_3R) are suggested to be beneficial for the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. The H_3R activates $G_{i/o}$-proteins to inhibit adenylyl cyclase activity and modulates phospholipase A_2 and MAPK activity. Herein we show that in transfected SK-N-MC cells the H_3R modulates the activity of the Akt/Glycogen synthase kinase 3β (GSK-3β) axis both in a constitutive and agonist-dependent fashion. H_3R stimulation with the H_3R agonist immepip induces the phosphorylation of both Ser^{473} and Thr^{308} on Akt, a serine/threonine kinase that is important for neuronal development and function. The H_3R-mediated activation of Akt can be inhibited by the H_3R inverse agonist thioperamide, and by wortmannin, LY294002 and PTX, suggesting the observed Akt activation occurs via a $G_{i/o}$-mediated activation of phosphoinositide-3-kinase. H_3R activation also results in the phosphorylation of Ser^{9} on GSK-3β, which acts downstream of Akt and has a prominent role in brain function. In addition, we show the H_3R-mediated phosphorylation of Akt at Ser^{473} to occur in primary rat cortical neurons and in rat brain slices. The discovery of this signaling property of the H_3R adds new understanding to the roles of histamine and the H_3R in brain function and pathology.

Introduction

Histamine is involved in the regulation of numerous functions of the central nervous system, including arousal, cognition, locomotor activity, autonomic and vestibular functions, feeding and drinking, sexual behavior, and analgesia. Histamine-containing cell bodies, located in the tuberomammillary nucleus of the posterior hypothalamus, project to most cerebral areas in rodent and human brain. The histamine H_3 receptor (H_3R) was originally discovered on histaminergic neurons as a presynaptic autoreceptor regulating the release and synthesis of histamine. Subsequently, the H_3R was found to regulate the release of other neurotransmitters, including acetylcholine, dopamine, glutamate, noradrenalin, and serotonin. Consequently, H_3R specific ligands show potential therapeutic effects in models of obesity, epilepsy and cognitive diseases such as Alzheimer's disease and attention deficit hyperactivity disorder (ADHD) (Hancock, 2003; Passani et al., 2004; Leurs et al., 2005).

The cloning of the H_3R gene allowed detailed studies of the molecular properties of the receptor and indicated that the H_3R can activate several signal transduction pathways including $G_{i/o}$-dependent inhibition of AC, the activation of PLA_2 and MAP kinase as well as the inhibition of the Na^+/H^+ exchanger and inhibition of K^+-

induced Ca^{2+} mobilization (Leurs et al., 2005). In view of the interest in the H_3R as a potential therapeutic target, we have evaluated additional signaling properties of the H_3R heterologously expressed in SK-N-MC neuroblastoma cells (Lovenberg et al., 1999).

Unraveling the signal transduction cascades that are activated by the H_3R is essential in order to elucidate the molecular mechanisms underlying the potential H_3R-mediated modulation of brain function. Recently a lot of interest has been shown for signal transduction in the CNS via activation of the serine/threonine protein kinase B (PKB), also known as Akt (Brazil et al., 2004). A key substrate of Akt is the ubiquitously expressed protein serine-threonine kinase, glycogen synthase kinase-3 (GSK-3) (Jope and Johnson, 2004). In the CNS, the Akt/GSK-3β axis plays a prominent role in brain function and has been implicated in Alzheimer's disease and neurological disorders (Li et al., 2002; Rickle et al., 2004). Herein, we investigated the ability of the H_3R to modulate the activity of this Akt/GSK-3β axis. Our results indicate that the H_3R modulates the activity of the Akt/GSK-3β axis and we have uncovered a heretofore-unknown signaling mechanism of the H_3R that may aid in our understanding of the actions of H_3R ligands in models of CNS disease.

Results

Pharmacological characterization of the human H_3R stably expressed in SK-N-MC cells

The human H_3R stably expressed in SK-N-MC neuroblastoma cells was characterized by N^{α}-[methyl-^{3}H]-histamine binding. Saturation analysis revealed the presence of a single high affinity binding site exhibiting a K_d value of 1.1 ± 0.1 nM (n=4) and a B_{max} of 0.39 ± 0.05 pmol/mg protein (n=4) for the radioligand (Figure 1A). A selection of reference H_3R ligands was tested for their ability to compete with N^{α}-[methyl-^{3}H]-histamine (Figure 1A). Immepip (pK_i = 9.4 ± 0.1 n=5), histamine (pK_i = 8.4 ± 0.2 n=3) and thioperamide (pK_i = 6.9 ± 0.1 n=4) all competed for N^{α}-[methyl-^{3}H]-histamine binding to the human H_3R according to the expected pharmacology (Lovenberg et al., 1999) (Figure 1B).

Additionally, we confirmed the ability of the constitutively active H_3R (Morisset et al., 2000; Wieland et al., 2001) to modulate cAMP signaling. Immepip (pEC_{50}=9.9 ± 0.1 n=3) inhibited the forskolin (1 μM) induced accumulation of cAMP by 79 ± 10% (n=3). Thioperamide significantly increased forskolin-induced cAMP accumulation to 130 ± 6% with a pEC_{50} value of 7.1 ± 0.2 (n=3) (Figure 1C), confirming the previously reported constitutive H_3R activity in the transfected SK-N-MC cells (Wieland et al., 2001).

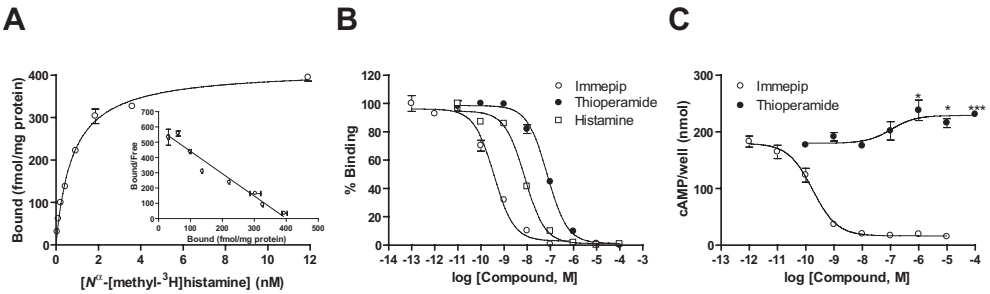

Figure 15. Pharmacological characterization of the human histamine H_3R heterologously expressed in SK-N-MC cells. (A) Saturation isotherm and Scatchard transformation (inset) of N^{α}-[methyl-^{3}H]-histamine binding to H_3R-expressing SK-N-MC cells. (B) Competition binding of N^{α}-[methyl-^{3}H]-histamine (0.7 nM) in the presence of immepip (○), thioperamide (●) and histamine (□). (C) Modulation of 10 μM forskolin-induced cAMP accumulation by the H_3R agonist immepip (○) and the inverse H_3R agonist thioperamide (●). Results represent the mean ± S.E.M. of three independent experiments performed in triplicate. * p<0.05, *** p<0.001 *versus* control.

H_3R-mediated phosphorylation of Akt

In view of the important role of Akt in activation of neuronal function, we evaluated the effects of H_3R activation on the phosphorylation of Akt. Phosphorylation of Akt at Ser^{473} and Thr^{308} are essential for its activation (Brazil et al., 2004). An antibody that specifically recognizes phosphorylation of Ser^{473} on Akt1, Akt2 and Akt3, is often used as a measure of Akt activation (Alessi et al., 1996). To determine if H_3R activation leads to Akt phosphorylation, H_3R-expressing cells were incubated with 10 nM immepip and the cells lysates were subsequently analyzed by specific anti-phospho-Akt immunoblots (Figure 2A). Control cells show a low basal level of Akt phosphorylation. Stimulation with immepip leads to a time- and dose-dependent phosphorylation of Akt at Ser^{473} (Figure 2B), which peaks at approximately 10 minutes (270 ± 10% of basal; n=3, p < 0.01) and then gradually declines, but remains significantly elevated even after 60 minutes (220 ± 30%, n=3, p < 0.05, Figure 3B). Similar kinetics are found for the H_3R-mediated Akt phosphorylation at Thr^{308} (Figure 2B). Stimulation of H_3R-expressing cells with varying concentrations of immepip for 10 minutes induces Ser^{473} phosphorylation of Akt with a pEC_{50} value of 9.4 (Figure 2C, D). The immepip-induced phosphorylation of Akt at Ser^{473} was completely inhibited by co-incubation with 10 μM of the inverse H_3R agonist thioperamide. Stimulation of H_3R-expressing cells with thioperamide alone resulted in a time- and dose-dependent reduction of the basal Akt phosphorylation in these cells (37 ± 6% of basal, pEC_{50} = 7.0, Figure 2C,D), indicating constitutive H_3R-mediated activation of the Akt pathway. Furthermore, thioperamide had no effect on SK-N-MC cells not transfected with the H_3R (Figure 2C,D). Additionally, we

tested the H_3R specific non-imidazole compound A-331440 and a found a similar maximal reduction of the basal Akt phosphorylation as for thioperamide and a pEC_{50} value of 7.7 (Figure 2D), which is in good agreement with its known potency (Hancock et al., 2004a).

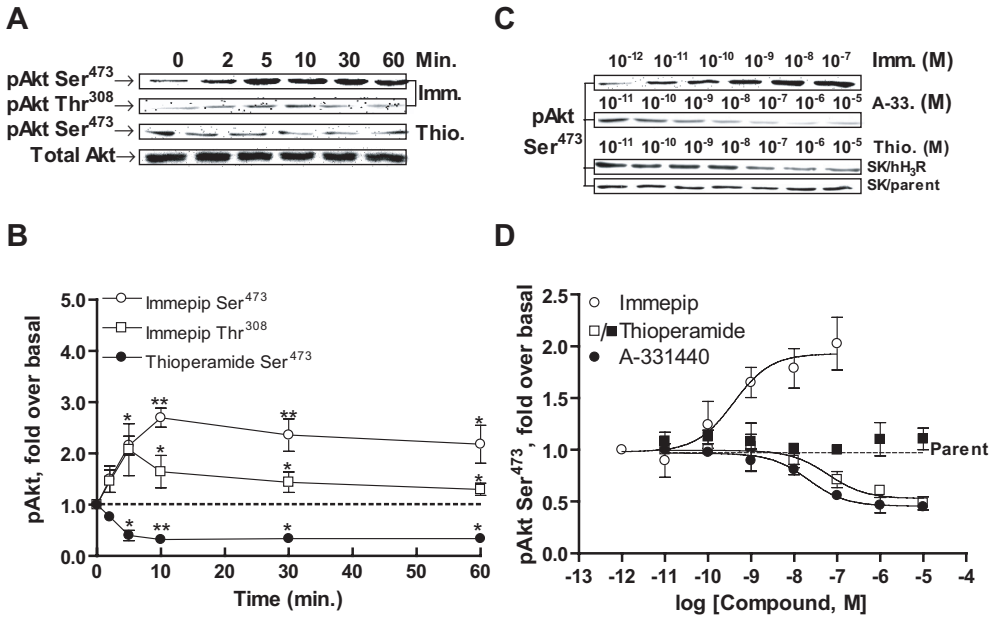

Figure 2. H_3R-mediated phosphorylation of Akt at either Ser^{473} or Thr^{308} in SK-N-MC cells. (A) and (B) Time-dependent phosphorylation of Akt on either Ser^{473} or Thr^{308} in H_3R-expressing SK-N-MC cells. Following serum starvation for 16 h, cells were treated with 10 nM immepip (○ and □) or 10 μM thioperamide (●) for the indicated time periods. (C) and (D) Effects of a 10 minute incubation with varying concentrations of either immepip (○), A-331440 (●) or thioperamide (□) on the phosphorylation of Akt at Ser^{473} in H_3R-expressing SK-N-MC cells with pEC_{50} values of 9.4, 7.7 and 7.0 respectively. Parental SK-N-MC cells not transfected with the H_3R stimulated for 10 minutes with thioperamide (■) showed no modulation of basal Akt phosphorylation at Ser^{473} (dashed line). (A and C) show a representative blot and (B and D) show the mean of the quantified data from three independent experiments ± S.E.M. * $p<0.05$, ** $p<0.01$ *versus* control.

We investigated the H_3R-mediated phosphorylation of Ser^{473} by several other histamine H_3R ligands tested at a concentration of 10 times their respective K_i values at both parental and SK-N-MC cells stably transfected with the H_3R. SK-N-MC cells stably transfected with the H_3R showed a higher basal Akt phosphorylation compared to the parental SK-N-MC cells, again indicating constitutive H_3R-mediated activation of the Akt pathway (Figure 3C, D).

Both the H_3R agonists, histamine and (*R*)-α-methylhistamine, as well as the proposed protean H_3R agonist proxyfan (Gbahou et al., 2003), significantly induced a H_3R-mediated Akt phosphorylation at Ser^{473} in H_3R expressing SK-N-MC cells (Figure 3A, B). Iodophenpropit, an inverse H_3R agonist (Wieland et al., 2001), was able to reduce constitutive H_3R-mediated Akt phosphorylation at Ser^{473} to 59 ± 20% of basal, similar to the effects of thioperamide. Some of these compounds were tested on SK-N-MC cells not transfected with the H_3R and were found not to modulate the Akt phosphorylation on Ser^{473} compared to basal (Figure 3C, D).

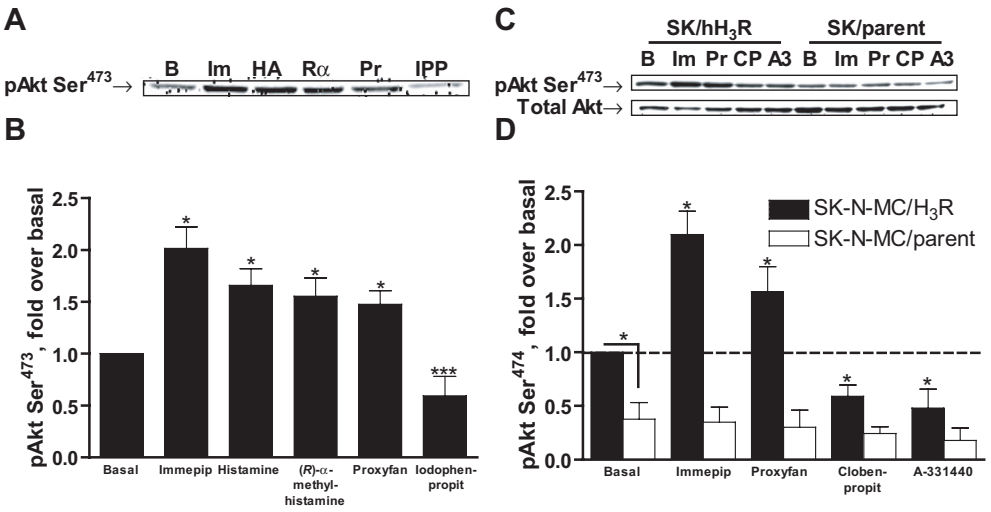

Figure 3. Phosphorylation of Akt at Ser^{473} in H_3R-expressing SK-N-MC cells by several H_3R ligands. (A) and (B) Effects of incubation for 10 minutes with either 10 nM immepip (Im), 100 nM histamine (HA), 10 nM (*R*)-α-methylhistamine (Rα), 100 nM proxyfan (Pr) or 10 nM iodophenpropit (IPP) on H_3R-mediated phosphorylation of Akt at Ser^{473}. (C) and (D) Comparison of SK-N-MC cells stably transfected with the H_3R (SK-N-MC/H_3R) and the parental cell line (SK-N-MC/parent) stimulated for 10 minutes with either 10 nM immepip (Im), 100 nM proxyfan (Pr), 10 nM clobenpropit (CP) or 1 µM A-331440 (A3). Significant modulation of the basal Akt phosphorylation of Akt Ser^{473} was observed in the SK-N-MC/H_3R cells, but not in the SK-N-MC/parent cells. Additionally, a significant increase in the basal phosphorylation of Akt on Ser^{473} was observed in the SK-N-MC/H_3R cells compared to the SK-N-MC/parent cells. (A) shows a representative blot and (B) shows the mean of the quantified data from three independent experiments ± S.E.M. * $p<0.05$, ** $p<0.01$ *versus* control.

H_3R-mediated Akt phosphorylation depends on $G_{i/o}$ proteins

We investigated the role of PTX-sensitive $G_{i/o}$ proteins in the H_3R-mediated phosphorylation of Akt at Ser^{473} by pre-incubation of the cells with 100 ng/ml PTX for 24 hours. Upon this pre-treatment, a slight increase in the basal Akt

phosphorylation at Ser^{473} was observed (120 ± 6% of basal, n=4) (Figure 4C, D). However, the PTX pre-treatment completely prevented the 10 nM immepip-induced H_3R-mediated phosphorylation of Akt at Ser^{473} (110 ± 10% of basal, n = 3). These observations indicate that the H_3R mediates phosphorylation of Akt at Ser^{473} in SK-N-MC cells involves PTX sensitive $G_{i/o}$-proteins.

H_3R-mediated Akt phosphorylation depends on PI3K

To test whether H_3R activation of Akt at Ser^{473} is dependent on the activation of PI3K, we used the PI3K inhibitors wortmannin and LY294002 at several concentrations. A one hour preincubation with either wortmannin or LY294002 dose dependently inhibited the 10 nM immepip-induced H_3R-mediated phosphorylation of Akt at Ser^{473} (120 ± 10%; n=4) (Figure 4A, B). These data indicate that H_3R activation results in Akt phosphorylation at Ser^{473} in SK-N-MC cells through a PI3K dependent pathway.

H_3R-mediated Akt phosphorylation is independent of Src/EGF receptor transactivation and MAP kinase activation

Modulation of PI3K/Akt signaling may occur via transactivation of growth factor receptors via the non-receptor tyrosine kinase Src (Thomas and Brugge, 1997). To investigate whether the H_3R-mediated Akt phosphorylation at Ser^{473} is mediated through the transactivation by the EGF receptor leading to activation of the Src family of tyrosine kinases we used AG1478 (250 nM) and PP2 (100 nM), potent and selective inhibitors of the EGF receptor and Src family tyrosine kinases, respectively (Figure 4C, D). Neither inhibitor had a substantial effect on either basal (AG1478: 81 ± 6%, n=3, and PP2: 80 ± 9%, n=3) or 10 nM immepip-induced Akt phosphorylation at Ser^{473} (AG1478: 170 ± 10%, n=3 and PP2: 180 ± 6%, n=3), indicating that the H_3R-induced phosphorylation of Akt at Ser^{473} is not due to transactivation of the EGF receptor.

The H_3R is known to activate the extracellular signal-regulated kinase 1/2 (Erk1/2) signaling pathway (Drutel et al., 2001). Activated Erk1/2 phosphorylates several mitogen-activated protein (MAP) kinase-activated protein (KAP) kinases (Chen et al., 2001), of which MAPKAPK2 is associated with Akt phosphorylation (Rane et al., 2001). In view of this potential 'cross-talk', we investigated if the H_3R-induced Akt phosphorylation at Ser^{473} involves MAP kinase signaling. H_3R-expressing cells were pre-incubated for 30 minutes with the specific MEK1/2 inhibitor U0126 (10 μM) to study the potential involvement of MAP kinase pathway in the Akt phosphorylation at Ser^{473}. Pre-treatment with U0126 had no effect on either basal (79 ± 20%; n=3) nor on the 10 nM immepip induced (180 ± 20%, n=2) Akt phosphorylation at Ser^{473} (Figure 4C, D).

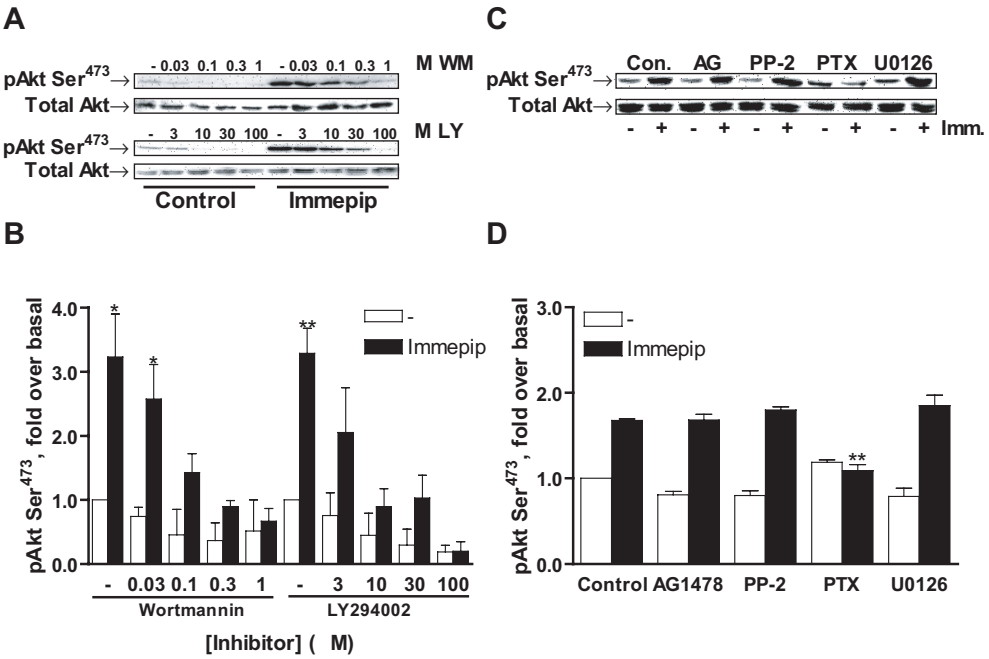

Figure 4. Effect of several inhibitors on the H_3R-mediated phosphorylation of Akt at Ser^{473} in H_3R-expressing SK-N-MC. (A) and (B) Effect of the PI3K inhibitors, wortmannin and LY294002 administered in increasing concentrations one hour prior to the stimulation with 10 nM immepip, on the H_3R-mediated phosphorylation of Akt at Ser^{473}. (C) and (D) Effects of the EGF-receptor inhibitor AG1478 (250 nM, 30 minutes), the Src family inhibitor PP-2 (100 nM, 30 minutes), the $G_{i/o}$-protein inhibitor PTX (100 ng/ml, 24 hours) and the MEK1/2 inhibitor U0126 (10 μM, 1 hour) on the immepip (10 nM) induced H_3R-mediated phosphorylation of Akt at Ser^{473}. (A and C) shows a representative blot and (B and D) shows the mean of the quantified data from three independent experiments ± S.E.M. *p<0.05, ** p<0.01 *versus* control.

GSK-3β is a target of H_3R signaling

GSK-3β is one of the main downstream targets of Akt (Cross et al., 1995). The GSK-3β kinase is active in resting cells whereas phosphorylation at Ser^9 by Akt leads to its inactivation (Stambolic and Woodgett, 1994). To examine whether activation of the H_3R results in phosphorylation of GSK-3β at Ser^9 we stimulated H_3R-expressing SK-N-MC cells with immepip and subsequently performed anti-phospho-GSK-3β immunoblots (Figure 5A). Cells incubated with 10 nM immepip showed a significant increase in Ser^9 phosphorylation of GSK-3β over control cells (Figure 5B). Immepip-induced H_3R-mediated phosphorylation of GSK-3β at Ser^9 peaks at approximately 30 minutes (170 ± 3%; n=3, p<0.01) and thereafter gradually declines, but remains significantly elevated even at 60 minutes (140 ±

20%, n=3, $p<0.05$). The immepip-induced phosphorylation of GSK-3β could be completely inhibited by co-incubation with 10 μM of the inverse H_3R agonist thioperamide (Figure 5B).

Immepip induces Ser^9 phosphorylation of GSK-3β in a dose dependent manner, with a pEC_{50} value of 9.5 (Figure 5C, D). Constitutive H_3R-mediated phosphorylation of GSK-3β at Ser^9 was shown by incubation with thioperamide alone, which results in a dose-dependent reduction of the constitutive phosphorylation of GSK-3β at Ser^9 with an estimated pEC_{50} value of 6.9 (Figure 5D), while thioperamide had no effect on SK-N-MC cells not transfected with the H_3R. Furthermore, the non-imidazole compound A-331440 also decreased the GSK-3β phosphorylation at Ser^9 to a similar extend as thioperamide with a pEC_{50} value of 7.9 (Figure 5D).

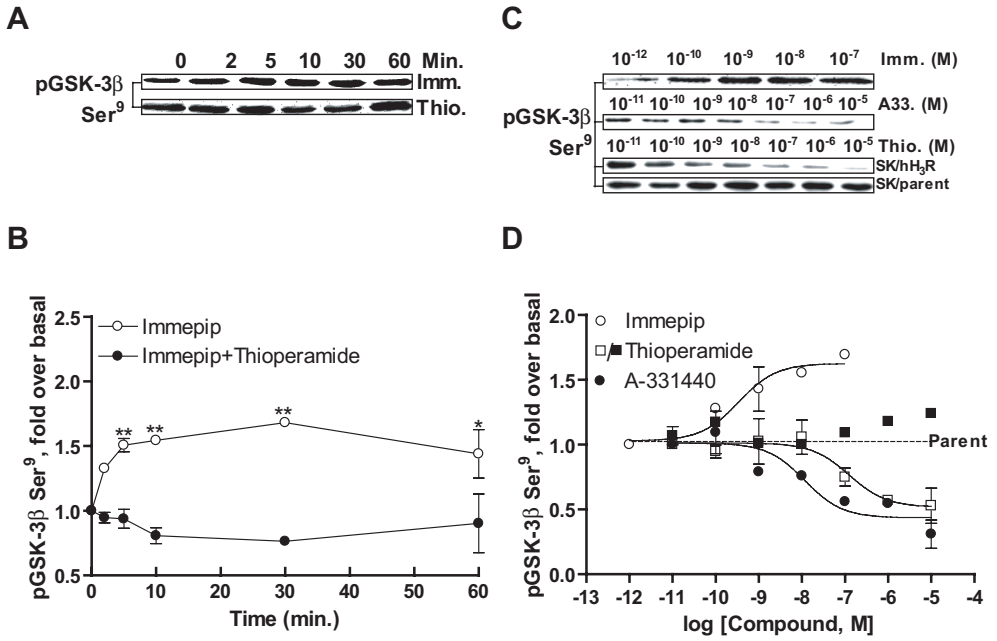

Figure 5. H_3R-mediated phosphorylation of GSK-3β at Ser9 in SK-N-MC cells. (A) and (B) Time-dependent phosphorylation of GSK-3β at Ser^9 in H_3R-expressing SK-N-MC cells. Following 16 h serum starvation, cells were treated with 10 nM immepip with (○) with or without 10 μM thioperamide (●) for the indicated time periods. (C) and (D) Effects of a 10 minute incubation with varying concentrations of either immepip (○), A-331440 (●) or thioperamide (□) on the phosphorylation of GSK-3β at Ser^9 in H_3R-expressing SK-N-MC cells with pEC_{50} values of 9.5, 7.9 and 6.9 respectively. Parental SK-N-MC cells not transfected with the H_3R stimulated for 10 minutes with thioperamide (■) showed no modulation of basal GSK-3β phosphorylation at Ser^9 (dashed line). (A and C) show a representative blot and (B and D) show the mean of the quantified data from three independent experiments ± S.E.M. * $p<0.05$, ** $p<0.01$ *versus* control.

H_3R-mediated phosphorylation of GSK-3β is Akt and $G_{i/o}$-protein dependent

As shown above, H_3R-mediated phosphorylation of Akt at Ser^{473} depends on $G_{i/o}$-proteins. To determine if the phosphorylation of GSK-3β at Ser^9 occurs through the same signaling mechanisms, we pre-incubated the cells with PTX. This completely blocked the H_3R-mediated GSK-3β phosphorylation at Ser^9 indicating the involvement of PTX sensitive $G_{i/o}$-proteins (Figure 6). Additionally, we evaluated whether the H_3R-mediated GSK-3β phosphorylation at Ser^9 was dependent on Akt activation (Figure 6). Therefore, we incubated H_3R-expressing cells with 10 μM of an Akt inhibitor (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) (Hu et al., 2000), 4 hours prior to stimulation with immepip.

Akt inhibition completely blocked the H_3R-mediated GSK-3β phosphorylation at Ser^9 (Figure 6).

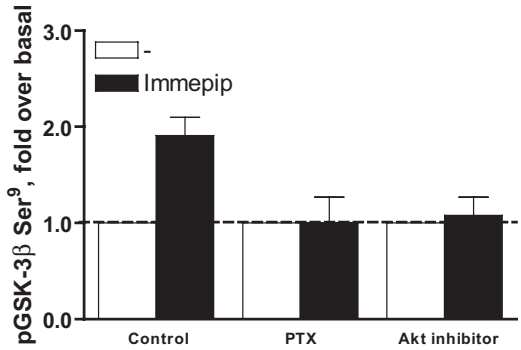

Figure 6. Effects of the the $G_{i/o}$-protein inhibitor PTX (100 ng/ml) and the Akt inhibitor 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonat, on the 10 nM immepip-induced H_3R-mediated phosphorylation of GSK-3β at Ser^9. The data shown are the mean of three independent experiments ± S.E.M. * $p<0.05$ *versus* control.

H_3R-mediated phosphorylation of Akt in rat cortical neurons

Primary cultures of cortical neurons were treated with H_3R ligands and analyzed by specific anti-phospho-Akt immunoblots. Both, 100 nM (*R*)-α-methylhistamine and 10 nM immepip produced a time-dependent significant increase in Akt phosphorylation at Ser^{473} in primary cultures of cortical neurons (420 ± 20% and 260 ± 40% respectively, Figure 7A, B). Co-incubation with 300 nM Thioperamide completely abolished the stimulatory effects of 100 nM (*R*)-α-methylhistamine (Figure 7B).

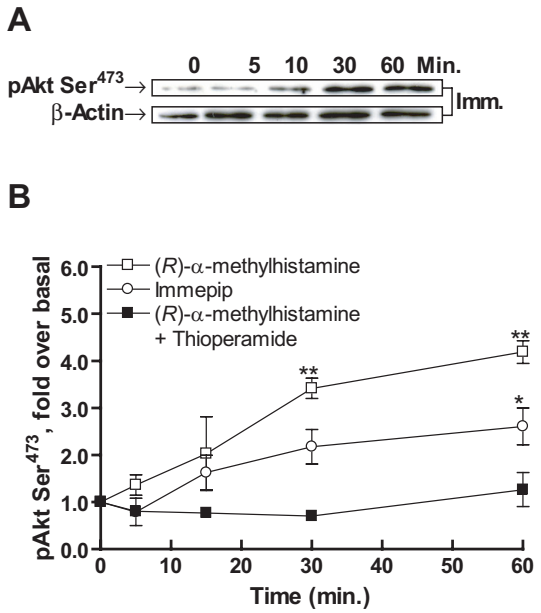

Figure 7. H_3R-mediated phosphorylation of Akt at Ser^{473} in primary cultures of cortical neurons. (A) and (B) Time-dependent phosphorylation of Akt at Ser^{473} Following serum starvation for 16 hr, cortical neurons were treated with either 100 nM (*R*)-α-methylhistamine (♦), 10 nM immepip (■), or (*R*)-α-methylhistamine and 300 nM thioperamide for the indicated time periods. (A) shows a representative blot and (B) shows the mean of the quantified data from three independent experiments ± S.E.M. **p*<0.05; ***p*<0.01 *versus* respective controls.

H_3R-mediated phosphorylation of Akt in rat striatal slices

Striatal slices of adult male 2-month-old Spraque-Dawley rats were incubated *ex vivo* for fifteen minutes with immepip and analyzed by specific anti-phospho-Akt immunoblots. Immepip produced a significant dose-dependent increase in Akt phosphorylation at Ser^{473} in rat striatal slices (Figure 8A, B) with a pEC_{50} of 9.6 and peaked at 100 nM (200 ± 20% of basal). Clobenpropit alone (100 µM, 100 ± 10%) had no effect on Akt phosphorylation at Ser^{473} in rat striatal slices, while it completely abolished the stimulatory effect of 100 nM immepip (100 ± 9%, Figure 8C).

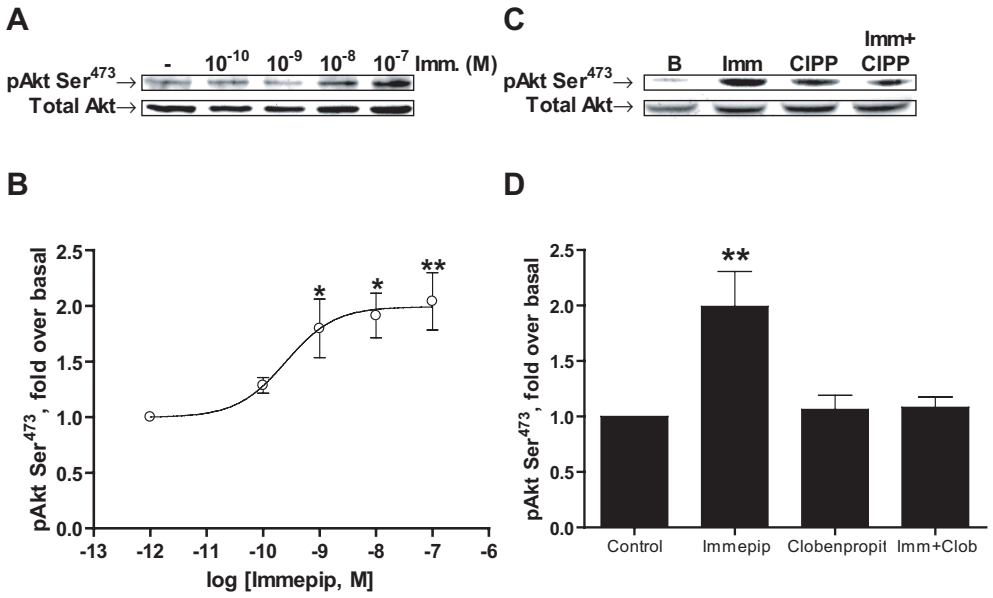

Figure 8. H_3R-mediated phosphorylation of Akt at Ser^{473} in rat striatal slices *ex vivo*. (A) and (B) Concentration dependent increase phosphorylation of Akt at Ser^{473} in rat striatal slices after treatment for 15 minutes with immepip (pEC_{50} = 9.6). Maximal phosphorylation was seen at 100 nM (204 ± 23% of basal). The data shown are the mean of five independent experiments ± S.E.M. (C) and (D) Akt phosphorylation at Ser^{473} in rat striatal slices after treatment with 100 nM immepip (199 ± 31% of basal), 100 µM clobenpropit (106 ± 13% of basal) or 100 nM immepip and 100 µM clobenpropit (108 ± 9% of basal). (A and C) show a representative blot and (B and D) show the mean of the quantified data from three independent experiments ± S.E.M. * p<0.05, ** p<0.01 *versus* control.

Discussion

After the cloning of the H_3R by Lovenberg et al. in 1999 significant efforts have been made to elucidate the signal transduction pathways activated by the H_3R. Using recombinant cell systems the H_3R has previously been shown to couple to several $G_{i/o}$-dependent signal transduction pathways, including the inhibition of AC, the activation of PLA_2, the stimulation of MAP kinase, the inhibition of the Na^+/H^+ exchanger and the inhibition of K^+-induced Ca^{2+} mobilization (Leurs et al., 2005).

In this study we have discovered a previously unknown signal transduction routes for the human 445 amino acid H_3R isoform (Lovenberg et al., 1999) after expression in SK-N-MC neuroblastoma cells at a relatively low level (0.39 pmol/mg protein). Upon expression in SK-N-MC cells, the human H_3R shows the expected pharmacological profile, as determined by the competition with N^{α}-[methyl-^{3}H]histamine, a radioligand selective for the H_3R. Moreover, the H_3R agonist

immepip potently inhibited the forskolin induced cAMP production in a PTX sensitive manner (data not shown), whereas the inverse H_3R agonist thioperamide increased the levels of forskolin induced cAMP. These findings are well in accordance with the known H_3R induced constitutive inhibition of adenylate cyclase (Morisset et al., 2000; Wieland et al., 2001) and all fit well with the known molecular pharmacology of the human H_3R. Besides the $G_{i/o}$-dependent inhibition of adenylate cyclase, we also show that H_3R activation can rapidly induce a sustained, $G_{i/o}$-dependent activation of the Akt/GSK-3β axis, which has recently been implicated in a variety of important CNS disorders, including Alzheimer's disease (Rickle et al., 2004). Akt activation was visualized by increases in phosphorylation of Akt at both Ser^{473} and Thr^{308}, using site-specific antibodies for the phosphorylated kinase. In SK-N-MC cells, H_3R activation leads to a subsequent inactivation of GSK-3β, which is a downstream target of Akt, as shown by the $G_{i/o}$- and Akt-activity dependent phosphorylation of Ser^{9} on GSK-3β. Interestingly, we find constitutive activation of the Akt/GSK-3β axis. Application of the inverse H_3R agonist thioperamide led to a time and concentration dependent decrease of basal Akt phosphorylation.

In H_3R-expressing SK-N-MC cells, the H_3R-mediated phosphorylation of Akt is independent of Src/EGF receptor transactivation and MAP kinase activation, but occurs via $G_{i/o}$-proteins and PI3K (Figure 4). Stimulation of PI3K upon H_3R activation likely dependents on G$\beta\gamma$-subunits from $G_{i/o}$ proteins, which are known to activate PI3K (Murga et al., 1998). Activated PI3K mediates the translocation of inactive Akt from the cytosol to the plasma membrane, where subsequent phosphorylation of Akt takes place at Ser^{473} and Thr^{308} in a PDK1 dependent manner (Brazil et al., 2004).. PKA has been shown to phosphorylate both Akt (Sable et al., 1997) and GSK-3β (Fang et al., 2000). Apparently, phosphorylation of Akt through PI3K upon H_3R stimulation occurs despite the Gα_i mediated inhibition of the cAMP formation, and subsequent inhibition of PKA.

The H_3R-mediated activation of Akt was not only observed in recombinantly expressing SK-N-MC cells, but also found to occur in primary cultures of cortical neurons and in striatal slices of Spraque-Dawley rats, although with somewhat slower kinetics. However, in both native systems constitutive H_3R-mediated activation of Akt was not observed. This apparent discrepancy between the recombinant cells and brain tissue can be explained by differential cellular context, as the H_3R was not over-expressed in the SK-N-MC cells (0.39 pmol/mg protein). Furthermore, this can also be explained by a difference between the human and rat H_3R, for which a lower level of constitutive activity has been observed when expressed SK-N-MC cells compared to the human H_3R (Wieland et al., 2001). Alternatively, as the H_3R expression was shown to correlate with its constitutive activity (Morisset et al., 2000), it might be that the expression in the native systems

is not high enough to observe a constitutive H_3R-mediated activation of the Akt pathway. The data with the endogenously expressed H_3R indicate that it is highly likely that this new signal transduction route is of physiological relevance and should be taken into account when analyzing H_3R-mediated modulation of CNS functions. Previously, the recognition of the MAPK pathway as a new signal transduction route for the H_3R in COS-7 cells (Drutel et al., 2001) has led to the discovery that H_3R activation indirectly induces MAPK activation in rat hippocampal CA3 cells. Moreover, this pathway has subsequently been convincingly linked to the effects of H_3R ligands on fear memory (Giovannini et al., 2003). At this moment, there is no information on the role of the H_3R-Akt pathway in brain function. Yet, in the CNS, the Akt/GSK-3β axis plays a prominent role in brain function and has been implicated in e.g. neuronal migration, protection against neuronal apoptosis (Brazil et al., 2004) and is believed to be altered in Alzheimer's disease, neurological disorders (Li et al., 2002; Rickle et al., 2004) and schizophrenia (Emamian et al., 2004). Because the CNS expression of the H_3R is high and restricted to specific areas of the brain during development (Karlstedt et al., 2003), one could speculate that the activation of Akt might be relevant for a H_3R-mediated neuronal migration during development of the CNS. It has been reported before that simultaneous MAPK and Akt activation are required for cortical neuron migration (Segarra et al., 2006). There is also some evidence that the H_3R plays a neuroprotective role in the CNS (Adachi et al., 1993) and that H_3R mRNA is upregulated in certain brain areas after e.g. induction of ischemia (Lozada et al., 2005) and kainic acid-induced seizures (Lintunen et al., 2005). Upregulation of the H_3R and the subsequent constitutive signaling to the Akt/GSK-3β pathway, as described, could be the mechanism by which the H_3R exerts its endogenous neuroprotective role.

The H_3R receptor has been proposed as potential drug target for the treatment of various important CNS disorders including Alzheimer's disease and schizophrenia (Leurs et al., 2005). For schizophrenic patients reduced levels of Akt protein were found (Emamian et al., 2004). Abnormal high levels and activity of GSK-3β are associated with neuronal death, paired helical filament tau formation and neurite retraction, as well as a decline in cognitive performance (Kaytor and Orr, 2002; Bhat et al., 2004). The present findings, elucidating the stimulation of the Akt/GSK-3β axis upon H_3R activation, seem not in line with the current general view on the therapeutic targeting of the H_3R. Especially H_3R antagonists are viewed as potential therapeutics for CNS disorders. To fully understand the impact of H_3R signaling to the Akt/GSK-3β axis in the CNS, more research will have to be performed in order to evaluate the (therapeutic) actions of H_3R ligands.

Materials and methods

Materials. Eagle's minimal essential medium, trypsin-EDTA, penicillin, nonessential amino acids, L-glutamine, streptomycin, and sodium-pyruvate were from Invitrogen (Invitrogen, Breda, The Netherlands) and fetal calf serum was from Integro (Zaandam, The Netherlands). Culture dishes were from Costar (Haarlemermeer, The Netherlands). All H_3R ligands were taken from laboratory stock or (re-)synthesized at the Vrije Universiteit Amsterdam. U0126 was obtained from Promega (Leiden, The Netherlands). Wortmannin, cyclic 3',5'-adenosine monophosphate (cAMP), pertussis toxin, A-331440 and the β-actin antibody were obtained from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). PP-2, AG1478 (N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine) and the Akt inhibitor (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) were obtained from Calbiochem (Merck Biosciences Ltd. Nottingham, UK). Antibodies recognizing Akt, phospho-Akt (Ser^{473} and Thr^{308}), phospho GSK-3β (Ser^{9}) and LY294002 were obtained from Cell Signaling (Cell Signalling Technology, Inc., Beverly, MA, USA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). G-418 was obtained from Duchefa (Duchefa Biochemie B.V., Haarlem, The Netherlands). 3-isobutyl-1-methylxanthine was obtained from Acros (Fischer Scientific, 's Hertogenbosch, The Netherlands). BSA fraction V was obtained from Fluka (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). [^{3}H]cyclic 3',5'-adenosine monophosphate ([^{3}H]cAMP, 40 Ci/mmol), was from Amersham ('s Hertogenbosch, The Netherlands); The enhanced-chemiluminescence assay "Western Lightning" and N^{α}-[methyl-^{3}H]histamine (85 Ci/mmol) was from PerkinElmer Life Sciences (Zaventem, Belgium).

Cell culture. SK-N-MC_H_3R cells (a kind gift from Dr. T.W. Lovenberg, R.W. Johnson Pharmaceutical Research Institute, San Diego, California, USA), a human neuroblastoma cell line that stably express the human H_3R (445-amino acid isoform) (Lovenberg et al., 1999) was maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM mixture of non-essential amino acids, 50 µg/ml sodium pyruvate, 50 IU/ml penicillin, and 50 µg/ml streptomycin in presence of 600 µg/ml G-418.

***N^{α}*-[methyl-^{3}H]histamine binding.** SK-N-MC_H_3R cells were scraped from their dishes; centrifuged (3 minutes, 1000 rpm) and the pellets were stored at -20°C until use. Before use, the pellets were dissolved in distilled water and homogenized for 2 seconds (40 Watt, Labsonic 1510). The cell homogenates (10-20 µg) were incubated for 60 minutes at 25°C with 0.6 nM N^{α}-[methyl-^{3}H]histamine (83.0 Ci/mmol) in 50 mM Tris (pH 7.4) with or without competing ligands in a total volume of 200 µl. The incubation was terminated by rapid filtration over polyethylenimine (0.3%) pretreated Unifilter GF/C filterplates with two subsequent washes with ice cold 50 mM Tris-HCl (pH 7.4). Radioactivity retained on the filter was determined by liquid scintillation counting on the Microbeta Trilux with 25 µl Microscint "O".

Measurement of cAMP. SK-N-MC cells stably expressing the H_3R were washed once with EMEM supplemented with 25 mM HEPES (pH 7.4 at 37°C) and preincubated in the same medium for 30 minutes at 37°C. Thereafter, 50 µl $5 \cdot 10^5$ cells were added per well to a 96-well plate containing the respective ligands in 50 µl EMEM supplemented with 0.3 mM 3-isobutyl-1-methylxanthine and 1 µM forskolin. After 10 minutes incubations were terminated by the addition of 20 µl 0.3 mg/ml saponin to each well to lyse the cells. Subsequently, cAMP levels were determined with a competitive protein kinase A (PKA) binding assay, as described (Wieland et al., 2001). Briefly, a PKA-containing fraction was isolated from bovine adrenal glands, which were homogenized in 10 volumes of 100 mM Tris-HCl, 250 mM NaCl, 10 mM EDTA, 0.25 M sucrose, and 0.1% 2-mercaptoethanol (pH 7.4 at 4 °C) and centrifuged for 60 min at 30.000 x g at 4°C. The supernatant, containing PKA, was carefully recovered and frozen in 1 ml aliquots at -80 °C. Before use, the PKA was diluted 15-fold in ice-cold phosphate buffer saline (PBS) and kept on ice. To each well 20 µl PBS with 0.6 nM [^{3}H]-cAMP (48.0 Ci/mmol) and 60 µl PBS with PKA was added. After 30 minutes the reaction was terminated by filtration over Unifilter GF/B filter plates with two subsequent washes with 200 µl ice cold 50 mM Tris-HCl (pH 7.4). Retained radioactivity was determined by liquid scintillation counting on the Microbeta Trilux with 50 µl Microscint "O". The amount of cAMP in each sample was calculated with GraphPad Prism version 4.01 for Windows, (GraphPad Software, San Diego, California, USA), using a generated standard cAMP curve (0.1 mM – 10 pM).

Western blot analysis. SK-N-MC cells stably expressing the human H_3R grown in 6-well plates ($5 \cdot 10^5$ cells/well) were serum starved overnight in serum free culture medium containing 0.1% BSA. Two hours before stimulation, the culture media was replaced with serum free culture medium containing 0.1% BSA (stimulation medium). Cells were stimulated with the indicated H_3R ligands. Incubations were stopped at the indicated times by aspiration of the stimulation medium, followed by a wash with PBS (pH 7.4 at 4°C). The cells were subsequently lysed in 100 µl radioimmunoprecipitation assay (RIPA) buffer (PBS containing 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 2 µg/ml aprotinin and 2 µg/ml leupeptin), sonicated, separated by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. The membrane was blocked for 60 minutes at RT in T-TBS (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 8.0) buffer containing 5% (v/w) milk powder. Subsequently, antibodies recognizing either Akt (1:1,000), or phospho-Akt (Ser^{473}) (1:1,000) were incubated overnight at 4°C in T-TBS containing 5% (v/w) BSA and used in combination with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000), incubated for 60 minutes at RT in T-TBS containing 5% (v/w) milk powder. The antibody recognizing β-actin (1:20,000) was incubated overnight at 4°C in T-TBS/5% BSA and used in combination with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000), incubated for 60 minutes at RT in T-TBS/5% Milk. Blots were stripped by incubation in 100 mM β-mercaptoethanol for 15-30 minutes at 50°C and rinsed multiple times in T/TBS before reuse. Immunoreactivity was detected by an enhanced-chemiluminescence assay and directly

quantified with a Kodak Image station (PerkinElmer Life and Analytical Sciences, Inc. Boston, MA USA).

Ex vivo analysis of Akt phosphorylation in rat.

Animals. Adult male 2-month-old Spraque-Dawley rats were used in this study. The experiments were approved by the Institutional Animal Care and Use Committee of Abo Akademi University and the Provincial State Office of Western Finland. The experiments were carried out in agreement with the ethical guidelines of the European Community Council Directives 86/609/EEC.

Stimulation of striatal slices. The rats were killed by decapitation, the brains were quickly removed and placed into ice-cold oxygenated medium of the following composition: 126 nM NaCl, 1.5 nM KCl, 1.25 nM KH_2PO_4, 26 nM $NaHCO_3$, 1.5 nM $MgSO_4$, 2 nM $CaCl_2$, and 10 nM D-glucose (pH 7.4). All following procedures were performed in the same medium under constant oxygenation with 95% O_2-5% CO_2. The forebrain was cut out and sectioned into 500 µm thick frontal slices using a vibratome and the striata were dissected from the slices, all in ice cold-medium, producing eight striatal slices per brain. The striatal slices were stabilized for 2 hr at room temperature and then transferred to incubation vials containing 1.5 ml of the same medium. The vials were transferred to 35°C for 45 min after which immepip and/or clobenpropit was added in volumes of 0.5 ml. Slices were incubated in the presence or absence of the ligands for 15 min, after which the slices were removed from the medium and quick-frozen in liquid nitrogen.

Western blot analysis. Frozen slices were thawed on ice and homogenated in approximately 10 w/v RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 nM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 25 µl Na_3VO_4, 25 µl NaF, 1 tablet/10 ml buffer Complete Mini Protease Inhibitor Coctail (Roche, Basel, Switzerland). Protein determination was performed using Bio-Rad (Hercules, CA) Protein Assay reagent. An appropriate volume of 6x Laemmli buffer was added to the homogenates, and samples were boiled for 4 min. Samples (30 µg protein per well) were loaded on a 10% SDS-polyacrylamide gel, resolved by electrophoresis, and blotted onto a nitrocellulose membrane (Hybond, Amersham Biosciences, Arlington Heights, IL). The membrane was blocked for 1hr at RT in TBS-T containing 5% bovine serum albumin (BSA) and then incubated over night at +4°C with anti phospho-Akt ($Ser4^{73}$) (Cell Signaling Technology, Beverly, MA) diluted 1:1000 in TBS-T containing 5% BSA. The membrane was washed with TBS-T (5 × 5 min) and incubated at RT for 1 hr with a horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (1:3000; Bio-Rad Hercules, CA). The membrane was washed with TBS-T (5 × 5 min) and immunoreactivity was detected using chemiluminescence (Amersham Biosciences ECL Western Blotting Analysis System). Blots were stripped by incubation in 0.1 M glycine (pH 2.5) for 20 min at RT and rinsed in Tris-HCl (2 × 5 min) and in TBS-T (2 × 5 min). The membrane was then blocked with 5% BSA in TBS-T, incubated with anti-Akt (Cell Signaling Technology, Beverly, MA) diluted 1:1000 at +4°C over night, incubated in secondary antibody, and developed as described above. Blots were scanned and analyzed using an MCID image analysis system (Imaging Research, St. Catherines, Ontario, Canada).

Analysis of Akt phosphorylation in primary cultures of cortical neurons.

Cell culture. Neuronal cortical cultures were prepared from ED17 fetal Sprague Dawley rats. Cerebral cortices were dissociated in sterile Dulbecco's phosphate buffer saline (D-PBS; Sigma-Aldrich, Milano, Italy), and neurons isolated in the same medium containing trypsine (0.5% in sterile D-PBS), at 37 ^{0}C for 10 min. After centrifugation, dissociated neurons were re-suspended in neurobasal medium (NBM; Gibco, Invitrogen Corporation) supplemented with 2% B-27 and 0.5 mM glutamine (both from Gibco), and then plated on 48-well plates coated with poly-D-lysine at a density of approximately 1.3 x 10^6/well. Cultures were maintained in NBM at 37 ^{0}C in a humidified, 5% CO_2 environment. In order to lower basal Akt phosphorylation, neurons were serum (B-27) deprived for approximately 16 hours before pharmacological treatments. Drugs were dissolved in sterile NBM and added to the cultures at the designated concentrations and for the length of time indicated.

Immunoblotting. Cells were harvested in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 µg/ml aprotinin and leupeptin, and 1% SDS). Proteins (20 µg) were separated using SDS-polyacryl-amide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA USA). Membranes were blocked for 1 h in PBS containing 0.1% Tween 20 (Sigma) and 5% skimmed milk (TBPS/5% milk; Biorad, Hercules, CA USA), and then probed overnight with primary antibodies against Akt phosphorylated at Ser^{473} (1:2000; Cell Signaling Technology, Beverly, MA; USA) in TPBS containing 1% Bovine Serum Albumin (Sigma). Membranes were then washed with TBPS and incubated for 1 h at room T in TBPS/5% milk containing anti-rabbit peroxidase-conjugated secondary antibody (1:5000; Pierce, Rockford, Il, USA). After washing in TBPS, ECL was used to visualize the peroxidase-coated bands, following the kit instructions. As a control, membranes were successively incubated in antibodies against actin (1:10,000 in TBPS/5% milk) for I h at room T. Membranes were then washed with TBPS and incubated for 1 h at room T in TBPS/5% milk containing anti-mouse peroxidase-conjugated secondary antibody (1:5000; Pierce) and developed with ECL as previously described.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, USA). Differences among means were evaluated by ANOVA, followed by the Dunnett's post test if indicated. For all analyses, the null hypothesis was rejected at the 0.05 level.

Chapter 7

Histamine H_3 receptor-mediated release of intracellular calcium via a $G_{i/o}$-phospholipase C dependent mechanism

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Abstract

In this study we investigated the effect of activation of histamine H_3 receptors, using stably transfected SK-N-MC neuroblastoma cells, on the intracellular calcium mobilization. We show that activation of the histamine H_3 receptor leads to a rapid and transient increase in intracellular calcium levels. Furthermore, we show that this histamine H_3 receptor-mediated release of intracellular calcium $[Ca^{2+}]_i$ is PTX sensitive, is independent of extracellular calcium, and does not involve ryanodine receptors. The H_3 receptor response originates from inositol 1,4,5-trisphosphate receptor sensitive stores, which are activated in a Gi/o protein and phospholipase C-dependent manner.

Introduction

Drugs targeting the histamine H_3 receptor are suggested to be beneficial for the treatment of e.g. obesity, myocardial ischemia and neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Hancock, 2003; Passani et al., 2004; Leurs et al., 2005). Detailed knowledge on the molecular aspects of H_3 receptor signaling is therefore of great interest in order to fully understand the cellular actions of potential new drugs that modulate histamine H_3 receptor activity. The histamine H_3 receptor is generally accepted to be a G_i-coupled GPCR inhibiting the activity of the membrane bound enzyme adenylyl cyclase (AC) (Lovenberg et al., 1999; Drutel et al., 2001). Moreover, H_3 receptor activation of G_i-proteins has been reported to affect a variety of other signal transduction pathways, including the activation of MAPK, arachidonic acid release and Akt/GSK-3β (for review see Leurs et al., 2005). Activation of the histamine H_3 receptor was also shown to inhibit voltage gated calcium channels, mostly through N-type channels (Oike et al., 1992; Endou et al., 1994; Poli et al., 1994; Takeshita et al., 1998). In SH-SY5Y cells the inhibition of voltage gated channels was shown to be dependent on the histamine H_3 receptor-mediated $G_{i/o}$-protein-dependent inhibition of adenylyl cyclase activity and the subsequent decrease in protein kinase A activity (Seyedi et al., 2005). The histamine H_3 receptor-mediated decrease in Ca^{2+} influx was shown to result in the inhibition of glutamate release (Molina-Hernandez et al., 2001), to attenuate the histamine synthesis (Torrent et al., 2005), and to affect the norepinephrine exocytosis from cardiac synaptosomes and SH-SY5Y cells transfected with the H_3 receptor (Seyedi et al., 2005). In contrast to these findings, Cogé et al. showed that activation of histamine H_3 receptor 445 amino acid isoform expressed in CHO cells resulted in the mobilization of $[Ca^{2+}]_i$. Although no mechanistic details were reported by Cogé et al. (Cogé et al., 2001), this finding is in line with the fact that several G_i-coupled GPCRs (Clapham and

Neer, 1997), including the homologous histamine H_4 receptor (H_4R) (Hofstra et al., 2003), can induce an increase in $[Ca^{2+}]_i$ via the release of $G\beta\gamma$ subunits.
In this study we show that in stably transfected SK-N-MC neuroblastoma cells the activation histamine H_3 receptor leads to an increase in $[Ca^{2+}]_i$. Furthermore, we show that the histamine H_3 receptor-mediated increase in $[Ca^{2+}]_i$ is PTX sensitive, not dependent on extracellular calcium or the involvement of ryanodine receptors, but originates from inositol 1,4,5-trisphosphate receptor sensitive stores in a phospholipase C-dependent manner.

Results

Histamine H_3 receptor-mediated intracellular calcium mobilization is $G_{i/o}$-protein dependent

Activation of the full length histamine H_3 receptor (445 isoform) stably expressed in SK-N-MC cells (B_{max} = 516 ± 23 fmol/mg of protein, Wieland et al., 2001) results in intracellular calcium ($[Ca^{2+}]_i$) mobilization as measured by Fluo-4 fluorometry. Stimulation of cells with 0.1 μM of the histamine H_3 receptor agonist immepip, resulted in a rapid increase in $[Ca^{2+}]_i$ that attained a maximum of 400 nM (2.5-fold over basal) after 15 seconds (Figure 1A). Calcium levels returned to basal levels within 60 seconds after the agonist application (Figure 1A). The histamine H_3 receptor-mediated increase in $[Ca^{2+}]_i$ was completely inhibited by a 10 minute pre-incubation with 10 μM of the histamine H_3 receptor inverse agonist thioperamide (Figure 1A). The immepip and (*R*)-α-methylhistamine responses were dose dependent, yielding a pEC_{50} values of 9.0 ± 0.2 and 8.3 ± 0.2, respectively (Figure 1B). Also, histamine, the endogenous histamine H_3 receptor agonist, induced a significant increase in $[Ca^{2+}]_i$, similar to immepip (Figure 1C). Cells were pre-treated with 100 ng/ml pertussis toxin (PTX) for 24 h investigate the involvement of $G_{i/o}$-proteins in the action of the H_3 receptor agonists. The PTX treatment completely prevented the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization (Figure 1C).

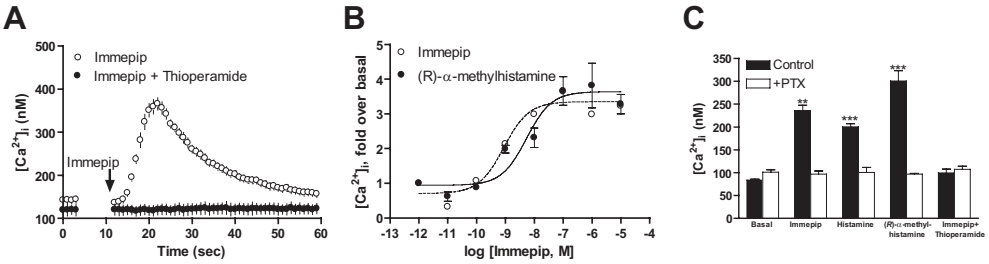

Figure 1. Pharmacological characterization of histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization in SK-N-MC cells. (A) Effects of immepip on intracellular calcium mobilization in histamine H_3 receptor-expressing SK-N-MC cells using Fluo-4,AM. (A) Stimulation with 0.1 μM immepip with (●) or without (○) 10 μM thioperamide. (B) Stimulation of the histamine H_3 receptor with the histamine H_3 receptor agonists immepip and (*R*)-α-methylhistamine leads to a dose dependent intracellular calcium mobilization. (C) Effect of PTX treatment (100 ng/ml, 24h) on calcium mobilization of several histamine H_3 receptor ligands. Concentrations used were 10 nM immepip, 100 nM histamine, 10 nM (*R*)-α-methylhistamine and 10 μM thioperamide. Results represent the mean ± S.E.M. of three independent experiments performed in triplicate. ** p<0.01, *** p<0.001 versus control.

Histamine H_3 receptor-mediated release of $[Ca^{2+}]_i$ originates from intracellular stores

To examine the role of extracellular calcium in the observed H_3 receptor response, Fluo-4 loaded SK-N-MC cells stably transfected with the histamine H_3 receptor, were incubated in Ca^{2+}-free buffer containing 2 mM EGTA. In the Ca^{2+}-free buffer the basal $[Ca^{2+}]_i$ gradually dropped by 65%. The addition of 0.1 μM immepip after one minute led to a 2.5-fold increase in $[Ca^{2+}]_i$ (Figure 2A). In contrast, cells exposed to 1 μM ATP did not show any increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, whereas in the presence of 1.3 mM extracellular free Ca^{2+} 1 μM ATP showed a 4-fold increase in $[Ca^{2+}]_i$ (Figure 2B).

To study the role of intracellular calcium in the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization, SK-N-MC cells were pre-loaded with BAPTA-AM or subjected to thapsigargin treatment. BAPTA-AM is a membrane-permeant precursor of BAPTA (Tsien, 1980), which is hydrolyzed by cytosolic esterases. The resulting BAPTA is thereby trapped intracellularly and will act as an intercellular Ca^{2+}-chelator. SK-N-MC cells pre-loaded with 20 μM BAPTA-AM for 1 hour before agonist stimulation did not show any histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization anymore (Figure 2C). Moreover, administration of 2.5 μM thapsigargin, which blocks Ca^{2+} re-uptake into the sarcoplasmic reticulum and endoplasmic reticulum (Ali et al., 1985) and thereby depletes the intracellular stores of Ca^{2+}, caused a 2.5-fold sustained increase in $[Ca^{2+}]_i$. Subsequent exposure of these

thapsigargin-treated cells to 0.1 μM immepip did not lead to an additional increase in $[Ca^{2+}]_i$ (Figure 2D).

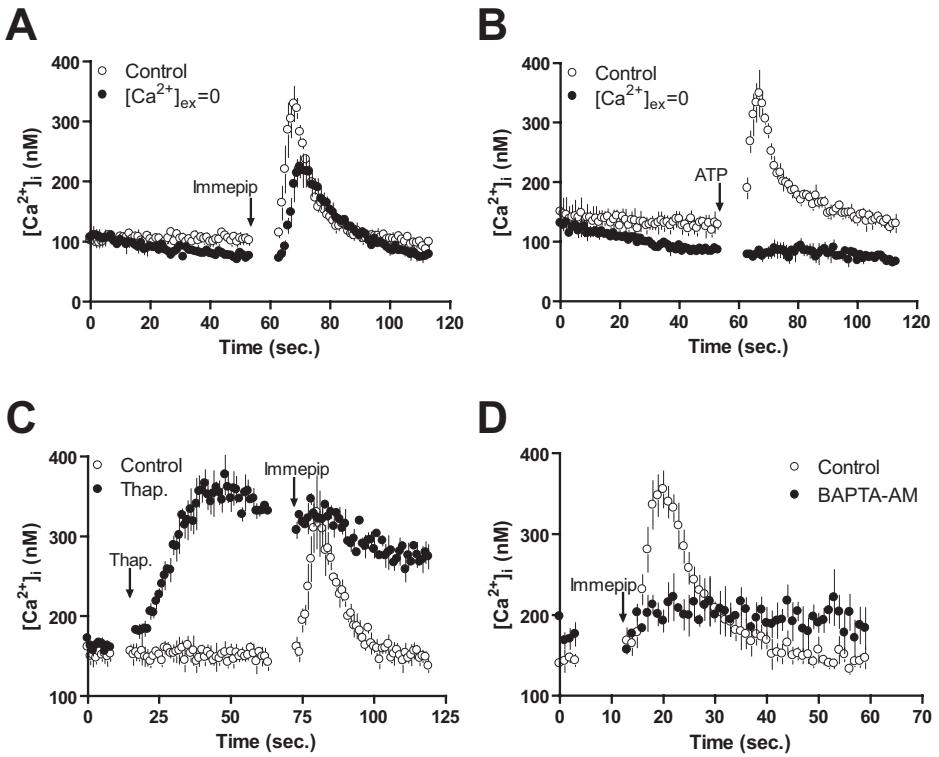

Figure 2. Histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization in SK-N-MC cells originates from intracellular stores. (A and B) Removal of extracellular calcium attenuates the histamine H_3 receptor mediated $[Ca^{2+}]_i$ mobilization. Cells were incubated in HBSS or Ca^{2+}-free HBSS containing 2 mM EGTA for one minute before the addition of 0.1 μM immepip (A) or 1 μM ATP (B). (C) Cells pre-treated with 20 μM BAPTA-AM before stimulation with 0.1 μM immepip completely attenuated the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization (D). Thapsigargin (2.5 μM) administered after 20 seconds caused an elevation in $[Ca^{2+}]_i$, administration of 0.1 μM immepip after 50 seconds did not further increase $[Ca^{2+}]_i$. Results represent the mean ± S.E.M. of three independent experiments performed in triplicate.

Histamine H_3 receptor-induced release of $[Ca^{2+}]_i$ depends on inositol 1,4,5-trisphosphate receptors and phospholipase C

A pre-incubation with 20 μM ruthenium red (Smith et al., 1988), a concentration sufficient to block ryanodine receptors (Ehrlich et al., 1994), 50 seconds before H_3 receptor stimulation led to a slight drop in basal $[Ca^{2+}]_i$, but did not affect the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization (Figure 3A).

To examine the role of inositol-1,4,5-trisphosphate receptors in the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization, we used 2-APB and 10 mM caffeine. Caffeine is known to activate ryanodine receptors and to inhibit PLC and 1,4,5-trisphosphate receptors (Ehrlich et al., 1994), whereas 2-APB is commonly used as a tool to study the involvement of inositol-1,4,5-trisphosphate receptors (Ehrlich et al., 1994). The application of 50 μM 2-APB or 10 mM caffeine before stimulation with immepip completely attenuated the histamine H_3 receptor-mediated release of $[Ca^{2+}]_i$ (Figure 3B, C). Additionally, application of 1 μM of the phospholipase C inhibitor U-73122 (Smith et al., 1990) completely blocked the histamine H_3 receptor-mediated rise in $[Ca^{2+}]_i$ (Figure 3D). Its inactive chemical control U-73343 (Smith et al., 1990) did not affect the histamine H_3 receptor-mediated increase in $[Ca^{2+}]_i$.

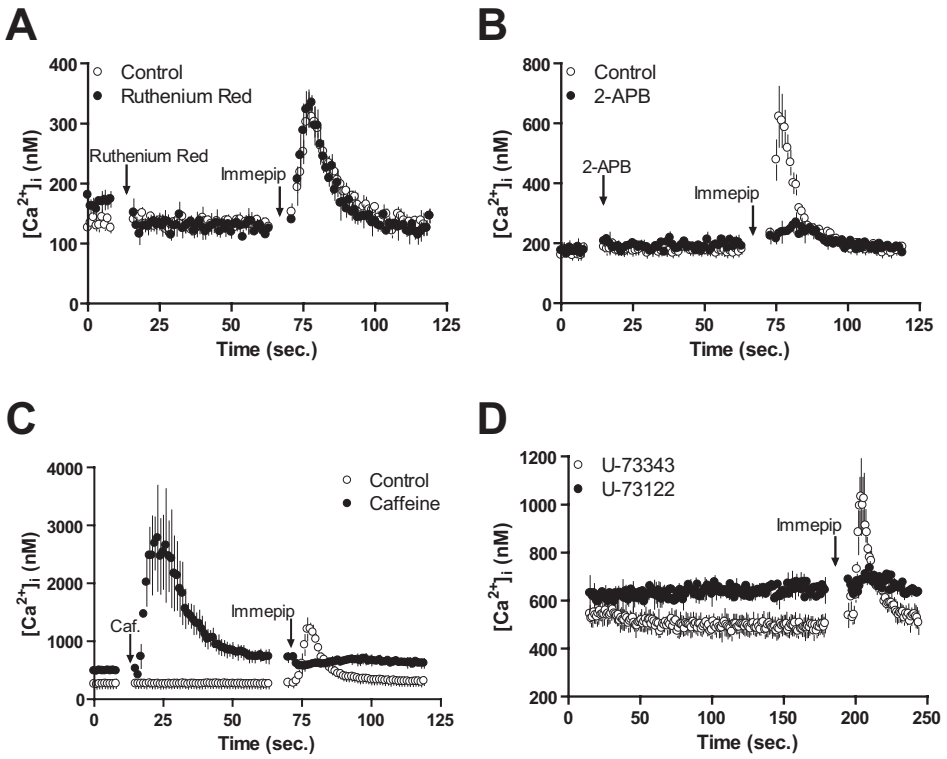

Figure 3. Histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization in SK-N-MC cells is inositol 1,4,5-trisphosphate receptor dependent. (A) The ryanodine receptor inhibitor 20 μM ruthenium red did not effect the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization. (B) 50 μM 2-APB (C) or 10 mM caffeine were administered 50 seconds before stimulation with 0.1 μM immepip and attenuated the histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization. (D) Cells pre-treated with a PLC inhibitor U-73122 or its inactive analogue U-73433 (both 1 μM) for 200 seconds before stimulation with 0.1 μM immepip, did not give a histamine H_3 receptor-mediated $[Ca^{2+}]_i$ mobilization. Results represent the mean ± S.E.M. of three independent experiments performed in triplicate.

Discussion

In this study we observe that histamine H_3 receptor activation leads to a rapid, but transient calcium mobilization from intracellular stores in stably transfected SK-N-MC neuroblastoma cells. The observed effect is PTX sensitive and resembles previous reported findings with e.g. the closely related $G\alpha_{i/o}$-coupled H_4 receptor, which mobilizes calcium in e.g. mast cells and eosinophils (Hofstra et al., 2003). The effects of histamine H_3 receptor activation on calcium mobilization are most likely mediated through $G\beta\gamma$ subunits from activated $G\alpha_{i/o}$-proteins as previously

reported for other GPCRs (Clapham and Neer, 1997). Activated Gβγ subunits can activate phospholipase C and result in the observed inositol-1,4,5-trisphosphate receptors dependent release of calcium from the endoplasmatic reticulum. Our present findings on the histamine H_3 receptor-mediated release of intracellular calcium are somewhat unexpected, since previously several studies reported inhibitory effects of histamine H_3 receptor activation on e.g. forskolin, 4-aminopyridine or K^+ induced calcium fluxes via the inhibition of the voltage-operated Ca^{2+} channels (Silver et al., 2002; Seyedi et al., 2005). The apparent discrepancy might be explained by the fact that activation of the histamine H_3 receptor leads to calcium mobilization from intracellular stores at basal levels $[Ca^{2+}]_i$, but inhibits voltage-operated Ca^{2+} channels at elevated $[Ca^{2+}]_i$. Moreover, one should also consider that H_3 receptor activation might differentially affect the calcium homeostasis in different cell types. Future studies should therefore investigate if similar findings are evident in cell types that endogenously express histamine H_3 receptors.

Materials and methods

Materials. Eagle's minimal essential medium (EMEM), trypsin-EDTA, penicillin, nonessential amino acids, L-glutamine, streptomycin, and sodium-pyruvate were from Invitrogen (Invitrogen, Breda, The Netherlands) and fetal calf serum was from Integro (Zaandam, The Netherlands). Culture dishes were from Costar. All histamine H_3 receptor ligands were taken from laboratory stock or (re-)synthesized at the Vrije Universiteit Amsterdam. Pertussis toxin, BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester), thapsigargin, probenecid, 2-aminoethoxydiphenyl borate (2-APB), U-73122, U73433, caffeine and ruthenium red were obtained from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). G-418 was obtained from Duchefa (Duchefa Biochemie B.V., Haarlem, The Netherlands). BSA fraction V was obtained from Fluka (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Fluo-4,AM and pluronic acid was obtained from Molecular Probes (Invitrogen BV, Breda, The Netherlands).

Cell culture. SK-N-MC cells, a human neuroblastoma cell line, stably expressing the 445-isoform of the human histamine H_3 receptor (a kind gift from Dr. T.W. Lovenberg, R.W. Johnson Pharmaceutical Research Institute, San Diego, California, USA (Lovenberg et al., 1999) were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere in EMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM mixture of non-essential amino acids, 50 μg/ml sodium pyruvate, 50 IU/ml penicillin, and 50 μg/ml streptomycin in presence of 600 μg/ml G-418.

Calcium mobilization assay. SK-N-MC cells stably expressing the histamine H_3 receptor cells were seeded at a density of $3 \cdot 10^4$ cells per well into clearbottom black 96-well microtiter

plates. The next day the culture medium was removed and replaced by 50 μl loading medium (Hanks Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 0.1% bovine serum albumin (BSA), 2.5 mM probenecid, 4 μM Fluo-4,AM and 0.05% pluronic acid, pH 7.4). Cells were loaded for 25 minutes at RT, washed twice with 100 μl buffer (HBSS containing 20 mM HEPES and 2.5 mM probenecid, pH 7.4) after which 180 μl of the same buffer was added. After 60 minutes the cells were stimulated and measured using a fluorometric imaging plate reader (Novostar, BMG) maintained at 37°C. The free Ca^{2+} concentration was calculated using the following formula: $[Ca^{2+}]_{free}=K_d(F-F_{min})/(F_{max}-F)$, where K_d is the dissociation constant of Fluo-4,AM for Ca^{2+} (345 nM; Molecular Probes), F is the fluorescence at intermediate calcium levels. F_{max} is the fluorescence of the calcium-saturated indicator determined by a 10 second measurement after the addition of 5% Triton X-100 in HBSS buffer and F_{min} is the fluorescence intensity of the indicator in absence of calcium as measure for 10 seconds after the addition of 100 mM EGTA in HBSS buffer. All measurements were done in triplicate and repeated at least three times.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, USA). Differences among means were evaluated by ANOVA, followed by the Dunnett's post test. For all analyses, the null hypothesis was rejected at the 0.05 level.

Chapter 8

Discussion and conclusions

Discussion

The research in this thesis describes the pharmacological characterization and novel signal transduction of the human histamine H_3 receptor (hH_3R) and its isoforms. In short, we characterized the constitutive activity of the hH_3R, investigated the pharmacological difference between two H_3R isoforms and studied the G-protein coupling specificity of four hH_3R isoforms. Additionally we describe characterization two signaling routes of the hH_3R, namely activation of the heretofore unknown hH_3R-mediated activation of the Akt/GSK-3β axis and characterization of the hH_3R-mediated release of intracellular calcium ($[Ca^{2+}]_i$).

Constitutive activity of the H_3R

The cloning of the hH_3R in 1999 by Lovenberg et al. and opened up to the possibility to recombinantly express the hH_3R in cell lines and thereby facilitated its pharmacological characterization (Lovenberg et al., 1999). Like many other G-protein coupled receptors (GPCRs) (Costa and Cotecchia, 2005; Bond and Ijzerman, 2006), both the histamine H_1 receptor and histamine H_2 receptor, were shown to be constitutively activity upon recombinant expression in cell lines (Alewijnse et al., 1998; Bakker et al., 2000). Establishing constitutive activity of a G-protein coupled receptor (GPCR) is of importance, because the level of constitutive activity determines if antagonists would behave as inverse agonists or neutral antagonists. Although the level of constitutive activity greatly depends on its expression and cellular context, pharmacological characterization of ligands enables one to rank the inverse agonists based potency and efficacy. Furthermore, some compounds are known to behave as neutral antagonists and these potentially have the additional benefit over inverse agonists as they are less likely to up-regulate the expression of constitutively active GPCRs as was shown for the histamine H_1, histamine H_2 and serotonin 5-HT_{2C} receptor. This up-regulation is accompanied by an increased responsiveness to agonists even at low expression of the receptor (Smit et al., 1996; Bakker et al., 2000; Devlin et al., 2004). These data indicate that even when constitutive activity is not be detected *in vivo*, treatment with an inverse agonists might still alter receptor expression and thereby change the cellular physiology and most notably the responsiveness to the endogenous agonists (Sadee et al., 2005; Salamone et al., 2007).

Stable expression of the hH_3R in SK-N-MC cells, a neuroblastoma cell line, at moderate expression levels (~300 fmol/mg protein) revealed that indeed, like the histamine H_1, H_2 and H_4 receptor, the hH_3R is constitutively active (Chapter 3, 6). In these cells we show that the hH_3R signals in a constitutively active manner towards

the AC pathway (Chapter 3) and the Akt/GSK-3β pathway (Chapter 6). Additionally many classical H_3R antagonists (e.g. thioperamide, clobenpropit, ciproxyfan) were shown to reverse this constitutive inhibition of AC in these SK-N-MC cells, as well as in stably transfected CHO (Morisset et al., 2000) and HEK293 (Wulff et al., 2002) cells and are in fact H_3R inverse agonists. Moreover, we found that VUF4904 acts as neutral antagonist in the AC signal transduction pathway (Chapter 3). More recently N-isopropylimpentamine and a propylene analogue of immepip, VUF5681, was shown to be a neutral antagonists for this pathway as well. These neutral H_3R antagonists competitively blocked the effect of H_3R agonists and H_3R inverse agonists (Kitbunnadaj et al., 2003). Moreover, VUF5681 was shown to block the effects of H_3R inverse agonist thioperamide on *in vivo* protein kinase A-mediated synthesis of histamine in rat brain cortex, whereas it did not modulate histamine synthesis (Moreno-Delgado et al., 2006b). Both these H_3R neutral antagonists have only small chemical differences compared to a H_3R agonist of inverse agonists. In Chapter 3 we show that small differences between H_3R ligands can bestow a compound to be either a agonists, neutral antagonist or inverse agonist. Neutral antagonists might be of particular importance for the H_3R as it was one of the few GPCRs for which constitutive activity has been shown to occur *in vivo* (Morisset et al., 2000; Adan and Kas, 2003). Therefore, it has been suggested that for the H_3R neutral antagonist would be preferred over inverse agonists in the treatment of cognitive disorders (Schwartz et al., 2003).

Besides stable expression of the hH_3R in SK-N-MC cells, we also stably expressed the hH_3R in C6 cells (rat glioma cell line, Chapter 4) and transiently in COS-7 cells (African green monkey kidney fibroblast cell line, Chapter 5). Also in these cell lines the hH_3R displayed constitutive activity. More importantly, both in the COS-7 cells and in the C6 cells we characterized the constitutive activity of some of the hH_3R isoforms and from these results it becomes clear that notably one splice variant of the hH_3R receptor is highly constitutively active, namely the $hH_3R(365)$. In Chapter 4 we describe that this constitutive activity of the $hH_3R(365)$ affects the hH_3R pharmacology and inverse agonists are less potent and have a lower affinity for this splice variant as can be predicted from the cubic ternary complex model for a GPCR with an increased constitutive activity. This is likely is of great importance *in vivo* as well because the $hH_3R(365)$ and the $hH_3R(445)$ are both highly expressed in the CNS, at least as shown in Chapter 4 by RT-PCR and by others (Cogé et al., 2001; Wellendorph et al., 2002). One might even speculate that the high constitutive activity of this particular splice variant is reason why *in vivo* constitutive activity of the H_3R could be shown (Morisset et al., 2000). In Chapter 4 we described the H_3R-mediated constitutive activity in a very direct manner by $[^{35}S]GTP\gamma S$ binding and on the second messenger level, namely the inhibition of AC by measurement of cAMP in a protein kinase A binding assay. Unpublished

observations show that in these C6 cells an increase in constitutive activity for the $hH_3R(365)$ occurs in phosphorylation of ERK1/2 as well and H_3R-mediated activation of the Akt/GSK-3β axis. In Chapter 5 we show the constitutive activity by transient expression of the hH_3R in COS-7 cells and use a downstream signaling event, namely activity of cAMP-Responsive Element (CRE) that in turn drives the expression of a luciferase reporter gene facilitating the read-out. Moreover, we also show that the G-protein, upon equal expression, does not affect the relative constitutive activity of the tested hH_3R isoforms. For the higly constitutively active $hH_3R(365)$, the level of spontanous activity is not determined by the expressed G-protein. The reason why the $hH_3R(365)$ shows this enhanced constitutive activity remains elusive, however the $hH_3R(329)$ nor the $hH_3R(415)$ show this increase in constitutive activity. Alignment of these hH_3R isoforms indicates that the C-terminal region of the IL3 might be important in this increased constitutive activity. This region has been implicated for a number of GPCR in the modulation of constitutive activity (Seifert and Wenzel-Seifert, 2002; Pauwels et al., 2003) and is generally attributed to in increased coupling to the G-proteins (Johnston and Siderovski, 2007a; Johnston and Siderovski, 2007b) or might be related to movement of the bottom of transmembrane domain VI in response to the binding of agonist ligands (Hubbell et al., 2003). Additionally, this region has been shown to be important for scaffolding protein spinophilin, providing an alternative explanation for the observed increase of constitutive activity in this splice variant of the hH_3R. Spinophilin was shown to recruit regulators of G-protein signaling (RGS)-proteins to the third intracellular loop of GPCRs (Wang et al., 2005). Sufficient amounts of RGS proteins have been shown to increases the fraction of basal GTPase activity contributed by the constitutive activity of a GPCR and to modulated the efficacy of inverse agonists (Hoffmann et al., 2001; Welsby et al., 2002). In intact cells, RGS proteins and possibly other modulators of G protein function are expected to regulate the constitutive activity of GPCRs and thus the likely therapeutic effectiveness of inverse agonists (Milligan, 2003).

hH_3R splice variants

The cloning of the hH_3R revealed that the full length receptor is GPCR 445 amino acids long (Lovenberg et al., 1999). Subsequently several papers reported on the discovery of several hH_3R isoforms and in two of these paerps the expression of the hH_3R isoforms was determined by either Northern blot analysis or RT-PCR. We determined the expression of the $hH_3R(445)$ and $hH_3R(365)$ by RT-PCR in Chapter 4 and confirmed earlier repots of high expression of the $hH_3R(445)$ and $hH_3R(365)$ in the cerebellum, caudate, hypothalamus, cerebrum and the thalamus (Cogé et al., 2001; Wellendorph et al., 2002). However, all these techniques detect mRNA

levels which do not always reflect actual protein levels. As long as no specific radioligands are developed, which will be a difficult task at any rate, the actual expression of the hH_3R isoforms awaits the confirmation by specific antibodies raised against the different hH_3R isoforms.

To date only two papers described the pharmacological characterization of some of these hH_3R isoforms. The paper by Cogé et al. describes the cloning of six hH_3R isoforms and the characterization of three of them, the $hH_3R(445)$, $hH_3R(431)$ and $hH_3R(365)$. The $hH_3R(431)$ could not be detected by radioligand binding, whereas both the $hH_3R(445)$ and $hH_3R(365)$ showed [^{125}I]Iodoproxyfan radioligand binding. The lack of radioligand binding for the $hH_3R(431)$ can likely be attributed to the deletion of 14 amino acids in second transmembrane domain that eliminates a structurally important proline residue characteristic for amine receptors (Ballesteros et al., 2001; Visiers et al., 2002). [^{125}I]Iodoproxyfan competition binding experiments revealed a similar pharmacology for the $hH_3R(445)$ and $hH_3R(365)$, however the $hH_3R(365)$ was unable to elicit a functional response in response to H_3R agonists, neither for the inhibition of AC, release of intracellular Ca^{2+}, nor in a [^{35}S]GTPγS binding assay. The second report on the pharmacological characterization of the hH_3R isoforms by Wellendorph et al. describes the characterization of six hH_3R isoforms, the $hH_3R(445)$, $hH_3R(373)$, $hH_3R(365)$, $hH_3R(309)$, $hH_3R(301)$ and the $hH_3R(220)$. From these only the $hH_3R(445)$, $hH_3R(365)$ and $hH_3R(373)$ show N^{α}-[methyl-^{3}H]-histamine radioligand binding and a functional response to H_3R compounds as measure in a R-SAT™ reporter assay. The $hH_3R(309)$, $hH_3R(301)$ and the $hH_3R(220)$ lack many important residues important for function and the overall structural organization of a GPCR that is hardly is surprising that these are non-functional (Kristiansen, 2004). However, as was shown for the non-functional rat H_3R isoforms lacking TM7 we cannot excluded an indirect functional role by modulation the activity of the functional hH_3R isoforms by acting in a dominant active manner (Bakker et al., 2006). As described in contrast to the paper of Cogé et al., the paper of Wellendorph et al. shows a functional response of the $hH_3R(365)$ in response to stimulation with a H_3R agonist. The discrepancy between these papers formed basis to perform a detailed pharmacological characterization of the highly expressed $hH_3R(445)$ and $hH_3R(365)$ isoforms and is described in Chapter 4. Our data are in agreement with the paper of Wellendorp et al. with regard to the fact that the $hH_3R(365)$ is able to elicit a response after stimulation with a H_3R agonist. However, as shown in Chapter 4, the response of the $hH_3R(365)$ is much lower than the $hH_3R(445)$. Like Wellendorp et al. we indeed find that agonists are much more potent at the $hH_3R(365)$. In addition we also tested a panel of inverse agonists and found that these were less potent at the $hH_3R(365)$. The decrease in potency for inverse agonists and increased potency for agonists agrees with the proposal set forward

in Chapter 4 that the $hH_3R(365)$ is a constitutively active splice variant of the hH_3R. This is corroborated by the fact that the basal activity of the $hH_3R(365)$ is increased compared to the $hH_3R(445)$ for both the inhibition of AC and in a [^{35}S]GTPγS binding assay. Furthermore, we show that this is not due to an increase in expression of the $hH_3R(365)$ as determined by radioligand binding with agonists N^{α}-[methyl-^{3}H]-histamine and the inverse agonist [^{3}H]A-349821. These finding might also have clinical applications because although H_3R inverse agonist are more potent at the $hH_3R(445)$, at sufficient concentrations the inverse agonists are much more efficacious in inhibiting the constitutive activity of the $hH_3R(365)$. However, currently it is not know if the constitutive activity of the $hH_3R(365)$ is a determinant in the overall H_3R pharmacology or whether it plays a major role *in vivo*. It might be interesting to pursue this question by generation of transgenic mice expressing either the $H_3R(445)$ or the $H_3R(365)$ conditionally in a $H_3R^{-/-}$ background to address questions of constitutive activity of the H_3R during development and with regard the responsiveness of the H_3R towards H_3R inverse agonists.

Many splice variant have been described for the hH_3R, with deletions in the N-terminal part, IL3 and even lacking complete TM-domains. Potentially the most interesting splice variants are the ones that have variations in the third intracellular loop, as this region has been implicated in the binding to various proteins (Abramow-Newerly et al., 2006b). One family of proteins associated with the third intracellular loop are the G-proteins, which have been the subject of investigation in Chapter 5 of this thesis. Although for some GPCRs altered pharmacology has been observed for isoforms that have deletions in the IL3, we did not see major changes in the agonist mediated pharmacology upon expression of the tested PTX-insensitive $G\alpha_{i/o}$-proteins However, its seems that pharmacology of the potency of H_3R inverse agonists depends on the expression of the $G\alpha_{i/o}$-proteins. At the moment no clear explanation is available for this observation. However, not much is know at the moment of the interaction with other proteins. Important effector proteins, like β-arrestin, spinophilin and possibly RGS proteins, are known to interact with the third intracellular loop. All these proteins might effect the signaling of the other by a direct competition for binding of an influencing the signaling of the other, as described for modulation of G-proteins GTPase activity by RGS proteins by either enhancing the GTPase activity via their regulators of G protein signaling domain or inhibiting it through their guanine nucleotide dissociation inhibitor domain (Mittal and Linder, 2004; Shu et al., 2007).

The β-arrestin mediated signal transduction of GPCRs has been shown to be important in the down-regulation of signaling and in the activation ERK1/2. The activation rat H_3R has been shown to lead to ERK1/2 phosphorylation and

unpublished observation have shown this to be the case for the hH_3R as well. To date nothing is known about the role of β-arrestin is this important signal transduction event. Additionally there might be differential regulation of β-arrestin mediated activation ERK1/2 by the different hH_3R isoforms as well as the potential difference in β-arrestin mediated down-regulation of hH_3R signaling, features that so far have not been investigated.

Besides the hH_3R isoforms with variations in the third intracellular loop other hH_3R isoforms have been described with modifications at the N-terminus, isoforms $hH_3R(409)$, $hH_3R(395)$, $hH_3R(379)$, $hH_3R(329b)$, $hH_3R(293)$ and $hH_3R(290)$. All these isoforms lack 36 amino acids at the N-terminus containing a N-glycosylation site. These isoforms are thus far not characterized and might be interesting to study as glycosylation at GPCRs has been found to be important in the stabilization dimers (Michineau et al., 2006) and for correct trafficking to the membrane (Rands et al., 1990; Servant et al., 1996; Lanctot et al., 2005). Additionally, the third intracellular loop is suggested by some to play a major role in the formation of dimers through the formation of coiled-coil structures and potentially complicates the hH_3R pharmacology of the hH_3R isoforms that have deletions in the third intracellular loop even more. In general not much is know about the dimerization of the hH_3R, but especially regarding the multitude of naturally occurring isoforms with deletions in the third intracellular loop provides an important tool to test the importance of regions in this loop and its role in dimerization.

As described the potential differential role of the hH_3R isoforms in signaling remains largely elusive and even more fundamentally nothing is know about regulation of the expression of the hH_3R isoforms. The $hH_3R(445)$ is shown to be highly up-regulated during developments suggesting it plays an important role during development (Karlstedt et al., 2003). It would be highly interesting to know whether there is a specific role for some of these hH_3R isoforms and how the pattern of hH_3R expression is regulated.

To study the role of these hH_3R isoforms it might be interesting to knock-in one of the hH_3R isoforms in a H_3R knock-out background. In mice however, the multitude of hH_3R isoforms is not found so far, a observation that might reflect the higher order of complexity in humans with respect to proteins being able to bind to the hH_3R needing differential signaling GPCRs. Studying these events in a more human setting is more difficult and as far a know no hH_3R isoform specific compounds are currently available.

hH_3R mediated signaling

Before the cloning of the hH_3R, its was suggested that the hH_3R might couple to the pertussis toxin (PTX) sensitive class of $G\alpha_{i/o}$-proteins (Clark et al., 1993; Endou

et al., 1994; Clark and Hill, 1995; Clark and Hill, 1996). Expression in cell lines after the cloning of the hH_3R confirmed its coupling to $G\alpha_{i/o}$-proteins and subsequently revealed at least three signaling pathways for the H_3R: $G\alpha_{i/o}$-mediated inhibition of AC (Lovenberg et al., 1999; Morisset et al., 2000; Cogé et al., 2001; Wieland et al., 2001; Gomez-Ramirez et al., 2002; Uveges et al., 2002; Sanchez-Lemus and Arias-Montano, 2004), stimulation of p44/p42 mitogen activated protein kinase (MAPK) pathway (Drutel et al., 2001) and coupling to neuronal Na^+/H^+ exchange (Silver et al., 2001). For some of these pathways the relevance in the H_3R mediated physiology still has to be established.

For the H_3R-mediated modulation of AC its and the subsequent change in cAMP levels has been shown to play a role in the synthesis of histamine and the exocytosis of of norepinephrine (Gomez-Ramirez et al., 2002; Seyedi et al., 2005; Torrent et al., 2005; Moreno-Delgado et al., 2006b). However, some studies have shown the inhibition of cholinergic neurotransmission in the guinea pig ileum and the release of norepinephrine from mouse cortex to be independent of AC (Poli et al., 1993; Schlicker et al., 1994a; Lee and Parsons, 2000). A less explored part of the AC pathway is the role of the cAMP binding-protein protein kinase A. Protein kinase A is know to play an important role in a range of biological responses including gene expression, synaptic plasticity, and behavior (Brandon et al., 1997).

In Chapter 6 we describe a novel hH_3R-mediated signal transduction, the activation of the Akt/GSK-3β axis, and investigated the hH_3R mediated release of intracellular calcium. We show that activation of the hH_3R in a neuroblastoma cell line, primary cultures of cortical neurons and in striatal slices of Spraque-Dawley rats activates the Akt/GSK-3β axis in a phospho-inositol-3-kinase dependent manner. Moreover, at least in a neuroblastoma cell line this occurs in a constitutively active manner as well. The exact role of this pathway in the H_3R-mediated physiology remains to be determined. In view of the predominantly CNS expression of the hH_3R and the fact that the Akt/GSK-3β axis is known to play a prominent role in brain function one might speculate this H_3R-mediated pathway to play an important role in neuronal migration or protection against neuronal apoptosis (Brazil et al., 2004). With respect to protection against apoptosis some papers claim that the H_3R might play a neuroprotective role in the CNS (Adachi et al., 1993). Additionally, H_3R mRNA is up-regulated in certain brain areas after induction of ischemia and kainic acid-induced seizures (Lintunen et al., 2005; Lozada et al., 2005). An interesting aspect not explored regarding the Akt/GSK-3β signaling is the described mediation of neurite outgrowth. Regarding the almost exclusive expression of the hH_3R in the central nervous system, its role on memory formation and its potential role in development, the investigation of the effect of the hH_3R on neurite outgrowth might be an interesting phenomenon to pursue.

Additionally, the Akt/GSK-3β axis is believed to be altered in Alzheimer's disease, neurological disorders and schizophrenia (Li et al., 2002; Emamian et al., 2004; Rickle et al., 2004). Clearly future research needs to be done to evaluate the merit of these signaling events *in vivo*. The c-Fos mediated signaling has been shown to be important for the hH_3R pharmacology of the different pharmacological classes of H_3R ligands in being active in models of obesity or memory (Hancock et al., 2006). It would be interesting to see if these ligands also discriminate between the H_3R-mediated signaling pathways know to be involved in memory process, the Akt/GSK-3β and the ERK1/2 signaling.

In Chapter 7 we show that activation of the hH_3R stably expressed in SK-N-MC cells results in a rapid and transient $G\alpha_{i/o}$-protein dependent calcium mobilization from intracellular stores. Furthermore, we show that this hH_3R-mediated release of intracellular calcium is independent of extracellular calcium, does not involve ryanodine receptors and most likely originates from inositol 1,4,5-trisphosphate receptor sensitive stores in a phospholipase C-dependent manner. For the related $G\alpha_{i/o}$-coupled histamine H_4 receptor a similar to calcium mobilization is shown in mast cells and eosinophils (Raible et al., 1994; Hofstra et al., 2003). In contrast, there are reports that activation of the H_3R reduces the K^+-induced intracellular calcium mobilization in SH-SY5Y cells. This phenomenon was linked to inhibitory effect of the H_3R on the norepinephrine exocytosis in these cells as well as in cardiac synaptosomes (Silver et al., 2002). Subsequently this H_3R-mediated inhibition of K^+-induced calcium mobilization was shown to be dependent on the H_3R-mediated inhibition of protein kinase A activity. Inhibition of proteins kinase leads to \decreased Ca^{2+} influx through voltage-operated Ca^{2+} channels (Seyedi et al., 2005). In contrast to the results described in Chapter 7 no effects on the intracellular Ca^{2+} levels were observed upon H_3R activation before the K^+-induced calcium release. Clearly more work has to be done to delineate the differences between these cells, or actually what is specific to these SK-N-MC cells, that can explain these contrasting observations. Additionally, it would be interesting to see whether our finding described in Chapter 7 can be seen in certain cell types *in vivo*.

With respect to non G-protein mediated signaling we developed and characterized a G-protein uncoupled mutant hH_3R that is able to bind ligands but does not signal to CRE anymore. This $R^{3.50}A$ mutants has been described for many other GPCR to uncouple the receptor of the G-proteins (Acharya and Karnik, 1996; Shibata et al., 1996; Lu et al., 1997; Scheer et al., 1997; Alewijnse et al., 2000; Scheer et al., 2000; Chung et al., 2002) and might be a valuable tool to investigate the heretofore unknown role of hH_3R mediated G-protein independent signaling. This residue is part of the conserved (D/E)RY motif C-terminal end of TM3 and have been shown to play an important roles in signaling for a large number of receptors (Flanagan, 2005; Rovati et al., 2007). The $R^{3.50}$ is one of the most conserved residues in

rhodopsin-like GPCRs R3.50 and is thought to be important in the formation of intramolecular interactions that constrain the receptor in either an inactive or activate conformation and mutation of this residue has been shown to impair G-protein coupling (Flanagan, 2005).

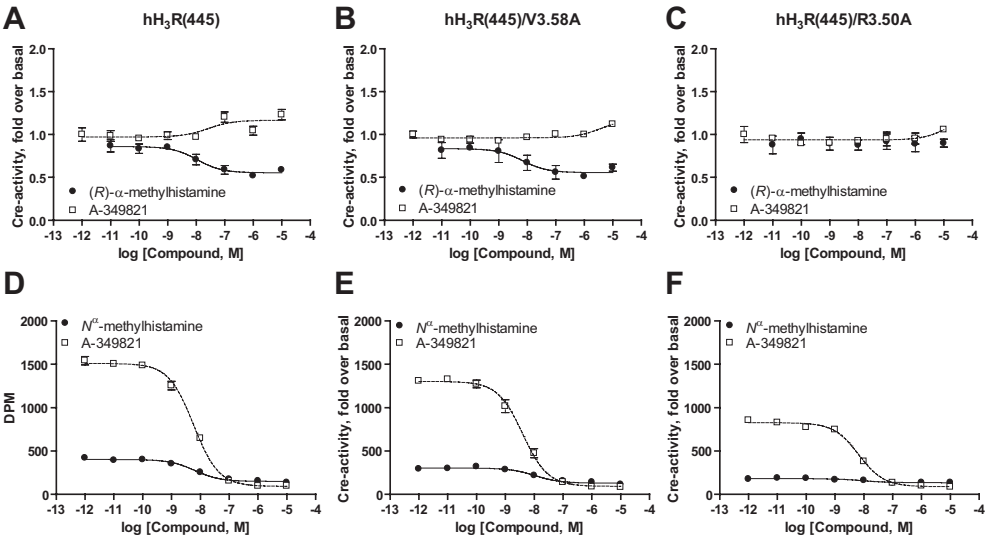

Figure 16. Uncoupling the hH_3R. Point mutation of $R^{3.50}A$ (C), but not $V^{3.58}A$ (B), prevents the H_3R-mediated modulation of CRE compared to wild-type (A), whereas, radioligand binding of both the agonists N^{α}-methylhistamine and the inverse agonists A-349821 is not markedly affected. These results suggest, as was shown for other GPCRs (for references, see text), that $R^{3.50}A$ mutation prevetents the H_3R from interacting with G-proteins.

Concluding remarks

Two members of the histamine receptor family, the histamine H_1 receptor and histamine H_2 receptor have proven to be a very lucrative drug target to develop specific ligands. The two remaining know histamine receptors, the H_3R and histamine H_4 receptor, still have to prove their merit. Especially for the H_3R a lot of pre-clinical evidence has recently been generating supporting the H_3R as an attractive drug target.

The H_3R has been pharmacologically almost xx years ago, but the cloning of the hH_3R has really pushed the field forward with the subsequent dedication of the pharmaceutical industry as well as ongoing efforts of academia. Clinical models of the hh_3R show that the compounds targeting the hH_3R might prove beneficial in several disease areas. However, although clinical trails are ongoing, to date nothing is known about its effect in human disease. To really prove the hH_3R as

being an attractive drug target the field is anxiously awaiting the outcome of these clinical trails.

The cloning of the hH_3R has led to the discovery of several signal transduction pathways that are modulated by the hH_3R. Some of these signaling pathways can be linked to relevant (patho)physiologies, like the hH_3R-mediated inhibition of the NHE. H_3R-mediated inhibition of NHE leads to a subsequent lowering in the exocytosis of norepinephrine and explaining the protective role of H_3R agonists during myocardial ischemia. For others like the Akt/GSK-3β axis described in this thesis future research will have to prove the exact role of this signaling event in the H_3R-mediated physiology.

During the time of this thesis the hH_3R has become and increasingly interesting drug-target as a consequence of the increase of *in vivo* data that became available on the efficacy of hH_3R compounds in clinical models. On the other hand, matters became more complicated with the discovery of over 20 hH_3R isoforms for which the individual roles remain elusive. Still, although the molecular aspect of the H_3R might have become more complex, the H_3R remains an attractive drug target. This claim is substantiated by the recent progress to clinical phase studies. The field is waiting for the outcome of these studies to once and for all show the therapeutic potential of H_3R inverse agonists and possibly even H_3R agonist.

Samenvatting

Signaal transductie van de histamine H_3 receptor

De histamine H_3 receptor wordt door velen beschouwd als een attractief drug-target voor de ontwikkeling van medicijnen voor de behandeling van vetzucht en een aantal cognitieve ziekten zoals alzheimer en ADHD. Het onderzoek in dit proefschrift beschrijft de farmacologische karakterisering van nieuwe signaaltransductie routes van de humane histamine H_3 receptor (hH_3R) en verschillende isovormen. In het kort, we hebben de constitutieve activiteit van de hH_3R en de farmacologische verschillen tussen twee H_3R isovormen gekarakteriseerd, evenals de specificiteit van de G-eiwit koppeling van vier hH_3R isovormen bestudeerd. Ook beschrijven we de karakterisering van twee nieuwe signaaltransductie routes van de hH_3R, namelijk de activatie van de Akt/GSK-3β as en de hH_3R-gemedieerde mobilisatie van intracellulair calcium ($[Ca^{2+}]_i$). De opgedane kennis in dit proefschrift draag bij aan het begrip omtrent de werking van de histamine H_3 receptor.

Het ontdekking van het hH_3R gen in 1999 door Lovenberg et al. opende de mogelijkheid tot het recombinant tot expressie brengen van de hH_3R in zoogdier cellijnen (Lovenberg et al., 1999). Zoals vele andere G-eiwit gekoppelde receptoren (GPCRs) (Costa and Cotecchia, 2005; Bond and Ijzerman, 2006) is ook aangetoond dat de histamine H_1 receptor en histamine H_2 receptor constitutief actief zijn als ze recombinant tot expressie worden gebracht in cellijnen (Alewijnse et al., 1998; Bakker et al., 2000). Het bepalen of een GPCR constitutief bepaalde signaal transductie routes activeerd is van belang, omdat het bepaald of antagonisten zich gedragen als een neutrale agonist of een inverse agonist. Een inverse agonist kan namelijk een extra effect teweeg brengen, namelijk het verhogen van de expressie van een GPCR en daardoor het effect van een endogene agonist verhogen (Smit et al., 1996; Bakker et al., 2000; Devlin et al, 2004; Sadee et al., 2005; Salamone et al., 2007).

Om te bepalen of de hH_3R constitutieve activiteit vertoond is deze GPCR stabiel tot expressie gebracht in een neuroblastoma cellijn (SK-N-MC cellen) en konden we laten zien dat ook de hH_3R constitutief actief is (Hoofdstuk 3, 6). De hH_3R signaleert in een constitutieve manier naar adenylyl cyclase (AC) (Hoofdstuk 3) en naar de Akt/GSK-3β as (Hoofdstuk 6). Tevens laten we zien dat vele klassiek H_3R antagonisten (b.v. thioperamide, clobenpropit, ciproxyfan) egenlijk inverse antagonisten zijn voor de hH_3R-gemedieerde signaaltransductie via AC. Ook hebben we een neutrale antagonist gevonden, namelijk VUF4904, en laten we zien dat kleine verandingen van dit molecuul kunnen leiden tot een agonist, neutrale antagonist of een inverse agonist (Hoofstuk 3). Neutrale antagonisten zijn mogelijk van extra belang in het hH_3R onderzoek aangezien de hH_3R een van de weinige

receptoren is waarvoor de constitutieve activiteit ook *in vivo* is aangetoond en derhalve een neutrale antagonist mogelijk de voorkeur verdient over een inverse agonist in de behandeling van cognitieve ziekten. (Morisset et al., 2000; Adan and Kas, 2003; Schwartz et al., 2003). De constitutieve activiteit van de hH_3R is niet alleen te meten wanneer de hH_3R recombinant tot expressie wordt gebracht in SK-N-MC cellen, maar ook in andere cellijnen zoals, C6 cellen (rat glioma cellijn, Hoofdstuk 4) en COS-7 cellen (Afrikaanse groene aap nier fibroblast cellijn, Hoofdstuk 5).

In deze laatste twee cellijnen vonden we dat een van de isovormen van de hH_3R, de $hH_3R(365)$, een hoge mate van constitutieve activiteit laat zien. We laten dit zien door middel van [^{35}S]GTPγS binding en door het meten van de 'second messenger' cAMP. In Hoofdstuk 4 beschrijven we dat de hoge constitutieve activiteit van de $hH_3R(365)$ de farmacologie beïnvloedt. We hebben gevonden dat inverse agonisten minder potent zijn en een lagere affiniteit hebben voor deze isovorm ten opzichte van de $hH_3R(445)$. Het omgekeerde geldt voor agonisten, m.a.w. een hogere functionele potentie en hogere bindingsaffiniteit. Dit is mogelijk ook *in vivo* van belang aangezien beide isovormen hoog tot expressie komen in het centrale zenuwstelsel (Hoofstuk 4).

In Hoofdstuk 5 gebruiken we een cAMP-reportergen methode voor het meten van de functionele respons van vier van de hH_3R isovormen. In deze assay wordt een lucifers-eiwit aangemaakt als het 'cAMP-responsive element' (CRE) wordt geactiveerd. Vervolgens wordt het substraat luciferine dat is toegevoegd aan de cellen omgezet in licht door het lucifers-eiwit; m.a.w. de hoeveelheid gemeten licht weerspiegeld de activiteit van de hH_3R. Deze techniek hebben we gebruikt om te onderzoeken wat de invloed van de verschillende G-eiwitten is op de farmacologie van de hH_3R en hoe G-eiwitten de farmacologie beïnvloeden van verschillende isovormen van de hH_3R die een deletie hebben in de derde intracellulaire loop (IL3). Voor de IL3 is aangetoond dat deze belangrijk is voor de koppeling van GPCR met de G-eiwitten. In Hoofdstuk 5 vergelijken we vier hH_3R isovormen, de $hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$ en $hH_3R(329)$; waarvan de laaste drie ten opzichte van de $hH_3R(445)$, deleties in de 3IL van respectievelijk, 30, 80 en 116 amino zuren. Van deze isovormen vertoond alleen de $hH_3R(365)$ een zeer hoge constitutieve activiteit en vooralsnog is ons onbekend waarom de $hH_3R(365)$ ten opzichte van de andere geteste isovormen zo constitutief actief is. Door het vergelijken van de eiwitsequentie weten we dat het C-terminale gedeelte in de 3IL waarschijnlijk van belang is. Aangetoond is dat dit gebied belangrijk kan zijn voor de koppeling van G-eiwitten, beweging van helix VI, maar ook voor de interactie met verschillende 'scaffold' eiwitten (Johnston and Siderovski, 2007a; Johnston and Siderovski, 2007b; Hubbell et al., 2003; Wang et al., 2005; Hoffmann et al., 2001; Welsby et al., 2002).

Het onderzoek naar de specificiteit in G-eiwit koppeling hebben we uitgevoerd door het tot expressie brengen van pertussis toxin (PTX)-ongevoelige G-eiwitten in COS-7 cellen. Hierdoor konden we alleen het door ons tot expressie gebrachte $G\alpha_{i/o}$-eiwit onderzoeken aangezien de endogene $G\alpha_{i/o}$-eiwitten kunnen worden geinactiveerd door PTX. Dit onderzoek beschreven in Hoofdstuk 5 leerde ons dat de verschillende geteste hH_3R isovormen ($hH_3R(445)$, $hH_3R(415)$, $hH_3R(365)$, $hH_3R(329)$) allemaal kunnen koppelen aan de geteste G-eiwitten, behalve de $hH_3R(329)$ voor welke we geen signaal konden meten met co-expressie van PTX-insensitieve $G\alpha_{i3}$-eiwit. Voor de agonist-gemedieerde response vonden we geen verschillen voor de verschillende G-eiwitten, echter voor de inverse agonist thioperamide laten we zien dat deze potenter is in de aanwezigheid van de PTX-insensitieve $G\alpha_{i2}$-eiwit en $G\alpha_{ob}$-eiwit.
In Hoofstuk 6 beschrijven we een nog niet eerder beschreven signaal transductie van de hH_3R, namelijk de activering van de Akt/GSK-3β as. Dit laten we zien in neuroblastoma cellijn (SK-N-MC cellen), primaire gekweekte cortical-neuronen en in geïsoleerde stukjes striatum van Spaque-Dawlley ratten. De activering van deze Akt/GSK-3β as is PI-3-kinase afhankelijk en in SK-N-MC cellen constitutief activeert door de hH_3R. Aangezien de modulatie van de Akt/GSK-3β as van belang is voor verschillende belangrijke functies van het brein, levert hoofdstuk 6 een belangrijke aanvulling omtrent onze kennis over de rol van de hH_3R in het brein.
In Hoofdstuk 7 laten we zien dat activering van de hH_3R in SK-N-MC cellen ook leidt tot een snelle $G\alpha_{i/o}$-afhankelijk vrijgave van intracellulair calcium uit inositol 1,4,5-trisphosphate gevoelige opslag. Tevens laten we zien dat dit onafhankelijk is van extracellulair calcium en ryanodine receptoren, maar afhankelijk is van phospholipase C.
Samenvattend, de hH_3R wordt door velen gezien als een attractief eiwit voor het genezen van verschillende ziekten, zoals vetzucht en verscheidene brein-gerelateerde ziekten, e.g. de ziekte van Alzheimer en ADHD. Het onderzoek is met name complex geworden door het bestaan van de verschillende hH_3R isovormen. Dit proefschrift draagt tot een beter begrip over de moleculaire eigenschappen van een aantal hH_3R isovormen, met name met betrekking tot de signaaltransductie.

References

Abramow-Newerly M, Ming H and Chidiac P (2006a) Modulation of subfamily B/R4 RGS protein function by 14-3-3 proteins. *Cell Signal* 18:2209-2222.

Abramow-Newerly M, Roy AA, Nunn C and Chidiac P (2006b) RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. *Cell Signal* 18:579-591.

Acharya S and Karnik SS (1996) Modulation of GDP release from transducin by the conserved Glu134-Arg135 sequence in rhodopsin. *J Biol Chem* 271:25406-25411.

Adachi N, Oishi R, Itano Y, Yamada T, Hirakawa M and Saeki K (1993) Aggravation of ischemic neuronal damage in the rat hippocampus by impairment of histaminergic neurotransmission. *Brain Res* 602:165-168.

Adan RA and Kas MJ (2003) Inverse agonism gains weight. *Trends Pharmacol Sci* 24:315-321.

Airaksinen MS, Paetau A, Paljarvi L, Reinikainen K, Riekkinen P, Suomalainen R and Panula P (1991) Histamine neurons in human hypothalamus: anatomy in normal and Alzheimer diseased brains. *Neuroscience* 44:465-481.

Aitken A (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways. *Trends Cell Biol* 6:341-347.

Aitken A, Baxter H, Dubois T, Clokie S, Mackie S, Mitchell K, Peden A and Zemlickova E (2002) Specificity of 14-3-3 isoform dimer interactions and phosphorylation. *Biochem Soc Trans* 30:351-360.

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P and Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo J* 15:6541-6551.

Alewijnse AE, Smit MJ, Hoffmann M, Verzijl D, Timmerman H and Leurs R (1998) Constitutive activity and structural instability of the wild-type human H_2 receptor. *J Neurochem* 71:799-807.

Alewijnse AE, Timmerman H, Jacobs EH, Smit MJ, Roovers E, Cotecchia S and Leurs R (2000) The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H(2) receptor. *Mol Pharmacol* 57:890-898.

Alguacil LF and Perez-Garcia C (2003) Histamine H3 receptor: a potential drug target for the treatment of central nervous system disorders. *Curr Drug Targets CNS Neurol Disord* 2:303-313.

Ali H, Christensen SB, Foreman JC, Pearce FL, Piotrowski W and Thastrup O (1985) The ability of thapsigargin and thapsigargicin to activate cells involved in the inflammatory response. *Br J Pharmacol* 85:705-712.

Allen PB, Ouimet CC and Greengard P (1997) Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc Natl Acad Sci U S A* 94:9956-9961.

Arrang JM, Drutel G and Schwartz JC (1995) Characterization of histamine H3 receptors regulating acetylcholine release in rat entorhinal cortex. *Br J Pharmacol* 114:1518-1522.

Arrang JM, Garbarg M, Lancelot JC, Lecomte JM, Pollard H, Robba M, Schunack W and Schwartz JC (1987) Highly potent and selective ligands for histamine H_3-receptors. *Nature* 327:117-123.

Arrang JM, Garbarg M and Schwartz JC (1983) Auto-inhibition of brain histamine release mediated by a novel class (H3) of histamine receptor. *Nature* 302:832-837.

Ash AS and Schild HO (1966) Receptors mediating some actions of histamine. *Br J Pharmacol Chemother* 27:427-439.

Avkiran M and Haworth RS (2003) Regulatory effects of G protein-coupled receptors on cardiac sarcolemmal Na+/H+ exchanger activity: signalling and significance. *Cardiovasc Res* 57:942-952.

Bakker RA (2004) Histamine H_3-receptor isoforms. *Inflamm Res* 53:509-516.

Bakker RA, Lozada AF, van Marle A, Shenton FC, Drutel G, Karlstedt K, Hoffmann M, Lintunen M, Yamamoto Y, van Rijn RM, Chazot PL, Panula P and Leurs R (2006) Discovery of naturally occurring splice variants of the rat histamine H_3 receptor that act as dominant-negative isoforms. *Mol Pharmacol* 69:1194-1206.

Bakker RA, Schoonus SB, Smit MJ, Timmerman H and Leurs R (2001) Histamine H(1)-receptor activation of nuclear factor-kappa B: roles for G beta gamma- and G alpha(q/11)-subunits in constitutive and agonist-mediated signaling. *Mol Pharmacol* 60:1133-1142.

Bakker RA, Wieland K, Timmerman H and Leurs R (2000) Constitutive activity of the histamine H(1) receptor reveals inverse agonism of histamine H(1) receptor antagonists. *Eur J Pharmacol* 387:R5-7.

Ballesteros JA, Shi L and Javitch JA (2001) Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. *Mol Pharmacol* 60:1-19.

Barbier AJ, Berridge C, Dugovic C, Laposky AD, Wilson SJ, Boggs J, Aluisio L, Lord B, Mazur C, Pudiak CM, Langlois X, Xiao W, Apodaca R, Carruthers NI and Lovenberg TW

(2004) Acute wake-promoting actions of JNJ-5207852, a novel, diamine-based H3 antagonist. *Br J Pharmacol* 143:649-661.

Benzing T, Kottgen M, Johnson M, Schermer B, Zentgraf H, Walz G and Kim E (2002) Interaction of 14-3-3 protein with regulator of G protein signaling 7 is dynamically regulated by tumor necrosis factor-alpha. *J Biol Chem* 277:32954-32962.

Benzing T, Yaffe MB, Arnould T, Sellin L, Schermer B, Schilling B, Schreiber R, Kunzelmann K, Leparc GG, Kim E and Walz G (2000) 14-3-3 interacts with regulator of G protein signaling proteins and modulates their activity. *J Biol Chem* 275:28167-28172.

Bermak JC, Li M, Bullock C and Zhou QY (2001) Regulation of transport of the dopamine D_1 receptor by a new membrane-associated ER protein. *Nat Cell Biol* 3:492-498.

Bertaccini G and Coruzzi G (1995) An update on histamine H3 receptors and gastrointestinal functions. *Dig Dis Sci* 40:2052-2063.

Bhat RV, Budd Haeberlein SL and Avila J (2004) Glycogen synthase kinase 3: a drug target for CNS therapies. *J Neurochem* 89:1313-1317.

Black JW, Duncan WA, Durant CJ, Ganellin CR and Parsons EM (1972a) Definition and antagonism of histamine H_2 -receptors. *Nature* 236:385-390.

Black JW, Duncan WA, Durant CJ, Ganellin CR and Parsons EM (1972b) Definition and antagonism of histamine H 2 -receptors. *Nature* 236:385-390.

Blandina P, Giorgetti M, Bartolini L, Cecchi M, Timmerman H, Leurs R, Pepeu G and Giovannini MG (1996) Inhibition of cortical acetylcholine release and cognitive performance by histamine H3 receptor activation in rats. *Br J Pharmacol* 119:1656-1664.

Bonaventure P, Letavic M, Dugovic C, Wilson S, Aluisio L, Pudiak C, Lord B, Mazur C, Kamme F, Nishino S, Carruthers N and Lovenberg T (2007) Histamine H3 receptor antagonists: from target identification to drug leads. *Biochem Pharmacol* 73:1084-1096.

Bond RA and Ijzerman AP (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci* 27:92-96.

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

Bongers G, Krueger KM, Miller TR, Baranowski JL, Estvander BR, Witte DG, Strakhova MI, van Meer P, Bakker RA, Cowart MD, Hancock AA, Esbenshade TA and Leurs R (2007b) A 80 amino acid deletion in the third intracellular loop of a naturally occurring human histamine H_3 isoform confers pharmacological differences and constitutive activity. *J Pharmacol Exp Ther*.

Bongers G, Sallmen T, Passani M, Mariottini C, Wendelin D, Lozada A, van Marle A, Navis M, Blandina P, Bakker R, Panula P and Leurs R (2006) New Signaling Pathways For The Histamine H_3 Receptor, in *XXXV Ann. Meet. Eur. Histamine Res. Soc*, Delphi.

Bovet D and Staub AM (1937) *Comp. rend. Soc. Biol.*:547.

Brady AE and Limbird LE (2002) G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. *Cell Signal* 14:297-309.

Brandon EP, Idzerda RL and McKnight GS (1997) PKA isoforms, neural pathways, and behaviour: making the connection. *Curr Opin Neurobiol* 7:397-403.

Brazil DP, Yang ZZ and Hemmings BA (2004) Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci* 29:233-242.

Bridges D and Moorhead GB (2005) 14-3-3 proteins: a number of functions for a numbered protein. *Sci STKE* 2005:re10.

Brown JD, O'Shaughnessy CT, Kilpatrick GJ, Scopes DI, Beswick P, Clitherow JW and Barnes JC (1996) Characterisation of the specific binding of the histamine H_3 receptor antagonist radioligand [3H]GR168320. *Eur J Pharmacol* 311:305-310.

Brown RE, Stevens DR and Haas HL (2001) The physiology of brain histamine. *Prog Neurobiol* 63:637-672.

Bunemann M, Frank M and Lohse MJ (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc Natl Acad Sci U S A* 100:16077-16082.

Burgaud JL and Oudart N (1993) Bronchodilatation of guinea-pig perfused bronchioles induced by the H_3-receptor for histamine: role of epithelium. *Br J Pharmacol* 109:960-966.

Cangioli I, Baldi E, Mannaioni PF, Bucherelli C, Blandina P and Passani MB (2002) Activation of histaminergic H3 receptors in the rat basolateral amygdala improves expression of fear memory and enhances acetylcholine release. *Eur J Neurosci* 16:521-528.

Cassar S (2000) Cloning of the guinea pig H_3 receptor. *Neuroreport* 11:L3-4.

Caulfield MP and Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 50:279-290.

Celanire S, Wijtmans M, Talaga P, Leurs R and de Esch IJ (2005) Keynote review: histamine H3 receptor antagonists reach out for the clinic. *Drug Discov Today* 10:1613-1627.

Chen G, Way J, Armour S, Watson C, Queen K, Jayawickreme CK, Chen WJ and Kenakin T (2000) Use of constitutive G protein-coupled receptor activity for drug discovery. *Mol Pharmacol* 57:125-134.

Chen J, Liu C and Lovenberg TW (2003) Molecular and pharmacological characterization of the mouse histamine H_3 receptor. *Eur J Pharmacol* 467:57-65.

Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C and Cobb MH (2001) MAP kinases. *Chem Rev* 101:2449-2476.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318:1258-1265.

Chu M, Huang ZL, Qu WM, Eguchi N, Yao MH and Urade Y (2004) Extracellular histamine level in the frontal cortex is positively correlated with the amount of wakefulness in rats. *Neurosci Res* 49:417-420.

Chung DA, Wade SM, Fowler CB, Woods DD, Abada PB, Mosberg HI and Neubig RR (2002) Mutagenesis and peptide analysis of the DRY motif in the alpha2A adrenergic receptor: evidence for alternate mechanisms in G protein-coupled receptors. *Biochem Biophys Res Commun* 293:1233-1241.

Clapham DE and Neer EJ (1997) G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol* 37:167-203.

Clapham J and Kilpatrick GJ (1992) Histamine H_3 receptors modulate the release of [^{3}H]-acetylcholine from slices of rat entorhinal cortex: evidence for the possible existence of H_3 receptor subtypes. *Br J Pharmacol* 107:919-923.

Clark AJ (1937) *General Pharmacology. In: Heffner's Handbuch der Experimentellen Pharmakologie Erganzungswerk Band 4*. Springer-Verlag, Berlin.

Clark EA and Hill SJ (1995) Differential effect of sodium ions and guanine nucleotides on the binding of thioperamide and clobenpropit to histamine H_3-receptors in rat cerebral cortical membranes. *Br J Pharmacol* 114:357-362.

Clark EA and Hill SJ (1996) Sensitivity of histamine H_3 receptor agonist-stimulated [^{35}S]GTP gamma[S] binding to pertussis toxin. *Eur J Pharmacol* 296:223-225.

Clark MA, Korte A and Egan RW (1993) Guanine nucleotides and pertussis toxin reduce the affinity of histamine H_3 receptors on AtT-20 cells. *Agents Actions* 40:129-134.

Cogé F, Guenin SP, Audinot V, Renouard-Try A, Beauverger P, Macia C, Ouvry C, Nagel N, Rique H, Boutin JA and Galizzi JP (2001) Genomic organization and characterization of splice variants of the human histamine H_3 receptor. *Biochem J* 355:279-288.

Coge F, Guenin SP, Rique H, Boutin JA and Galizzi JP (2001) Structure and expression of the human histamine H_4-receptor gene. *Biochem Biophys Res Commun* 284:301-309.

Costa T and Cotecchia S (2005) Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends Pharmacol Sci* 26:618-624.

Costa T, Lang J, Gless C and Herz A (1990) Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: specific regulation by antagonists and sodium ions. *Mol Pharmacol* 37:383-394.

Costa T, Ogino Y, Munson PJ, Onaran HO and Rodbard D (1992) Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol Pharmacol* 41:549-560.

Couve A, Kittler JT, Uren JM, Calver AR, Pangalos MN, Walsh FS and Moss SJ (2001) Association of GABA(B) receptors and members of the 14-3-3 family of signaling proteins. *Mol Cell Neurosci* 17:317-328.

Cowart M, Altenbach R, Black L, Faghih R, Zhao C and Hancock AA (2004) Medicinal chemistry and biological properties of non-imidazole histamine H3 antagonists. *Mini Rev Med Chem* 4:979-992.

Cowart M, Faghih R, Curtis MP, Gfesser GA, Bennani YL, Black LA, Pan L, Marsh KC, Sullivan JP, Esbenshade TA, Fox GB and Hancock AA (2005) 4-(2-[2-(2(R)-methylpyrrolidin-1-yl)ethyl]benzofuran-5-yl)benzonitrile and related 2-aminoethylbenzofuran H3 receptor antagonists potently enhance cognition and attention. *J Med Chem* 48:38-55.

Cross DA, Alessi DR, Cohen P, Andjelkovich M and Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785-789.

Cuatrecasas P (1974) Membrane receptors. *Annu Rev Biochem* 43:169-214.

Dale HH and Laidlaw PP (1910a) *J. Physiol.* 41:318-344.

Dale HH and Laidlaw PP (1910b) The physiological action of β-Imidazolethylamine. *J Physiol* 41:318-344.

de Almeida MA and Izquierdo I (1986) Memory facilitation by histamine. *Arch Int Pharmacodyn Ther* 283:193-198.

De Backer MD, Gommeren W, Moereels H, Nobels G, Van Gompel P, Leysen JE and Luyten WH (1993) Genomic cloning, heterologous expression and pharmacological characterization of a human histamine H_1 receptor. *Biochem Biophys Res Commun* 197:1601-1608.

De Backer MD, Loonen I, Verhasselt P, Neefs JM and Luyten WH (1998) Structure of the human histamine H_1 receptor gene. *Biochem J* 335 (Pt 3):663-670.

de Esch IJ, Thurmond RL, Jongejan A and Leurs R (2005) The histamine H_4 receptor as a new therapeutic target for inflammation. *Trends Pharmacol Sci* 26:462-469.

de Esch IJ, Timmerman H, Menge WM and Nederkoorn PH (2000) A qualitative model for the histamine H_3 receptor explaining agonistic and antagonistic activity simultaneously. *Arch Pharm (Weinheim)* 333:254-260.

De Lean A, Stadel JM and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J Biol Chem* 255:7108-7117.

Deakin M and Williams JG (1992) Histamine H2-receptor antagonists in peptic ulcer disease. Efficacy in healing peptic ulcers. *Drugs* 44:709-719.

Delaunois A, Gustin P, Garbarg M and Ansay M (1995) Modulation of acetylcholine, capsaicin and substance P effects by histamine H3 receptors in isolated perfused rabbit lungs. *Eur J Pharmacol* 277:243-250.

Dere E, De Souza-Silva MA, Topic B, Spieler RE, Haas HL and Huston JP (2003) Histidine-decarboxylase knockout mice show deficient nonreinforced episodic object memory, improved negatively reinforced water-maze performance, and increased neo- and ventro-striatal dopamine turnover. *Learn Mem* 10:510-519.

Devlin MG, Smith NJ, Ryan OM, Guida E, Sexton PM and Christopoulos A (2004) Regulation of serotonin 5-HT2C receptors by chronic ligand exposure. *Eur J Pharmacol* 498:59-69.

Ding W, Zou H, Dai J and Duan Z (2005) Combining restriction digestion and touchdown PCR permits detection of trace isoforms of histamine H_3 receptor. *Biotechniques* 39:841-845.

Dougherty MK and Morrison DK (2004) Unlocking the code of 14-3-3. *J Cell Sci* 117:1875-1884.

Drutel G, Peitsaro N, Karlstedt K, Wieland K, Smit MJ, Timmerman H, Panula P and Leurs R (2001) Identification of rat H_3 receptor isoforms with different brain expression and signaling properties. *Mol Pharmacol* 59:1-8.

Ehrlich BE, Kaftan E, Bezprozvannaya S and Bezprozvanny I (1994) The pharmacology of intracellular Ca(2+)-release channels. *Trends Pharmacol Sci* 15:145-149.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M and Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat Genet* 36:131-137.

Endou M, Poli E and Levi R (1994) Histamine H3-receptor signaling in the heart: possible involvement of Gi/Go proteins and N-type Ca++ channels. *J Pharmacol Exp Ther* 269:221-229.

Ericson H, Watanabe T and Kohler C (1987) Morphological analysis of the tuberomammillary nucleus in the rat brain: delineation of subgroups with antibody against L-histidine decarboxylase as a marker. *J Comp Neurol* 263:1-24.

Eriksson KS, Sergeeva O, Brown RE and Haas HL (2001) Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. *J Neurosci* 21:9273-9279.

Esbenshade TA, Fox GB and Cowart MD (2006a) Histamine H3 receptor antagonists: preclinical promise for treating obesity and cognitive disorders. *Mol Interv* 6:77-88, 59.

Esbenshade TA, Fox GB, Krueger KM, Baranowski JL, Miller TR, Kang CH, Denny LI, Witte DG, Yao BB, Pan JB, Faghih R, Bennani YL, Williams M and Hancock AA (2004) Pharmacological and behavioral properties of A-349821, a selective and potent human histamine H_3 receptor antagonist. *Biochem Pharmacol* 68:933-945.

Esbenshade TA, Fox GB, Krueger KM, Miller TR, Kang CH, Denny LI, Witte DG, Yao BB, Pan L, Wetter J, Marsh K, Bennani YL, Cowart MD, Sullivan JP and Hancock AA (2005) Pharmacological properties of ABT-239 [4-(2-{2-[(2R)-2-Methylpyrrolidinyl]ethyl}-benzofuran-5-yl)benzonitrile]: I. Potent and selective histamine H3 receptor antagonist with drug-like properties. *J Pharmacol Exp Ther* 313:165-175.

Esbenshade TA, Strakhova MI, Carr TL, Sharma R, Witte DG, Yao BB, Miller TR and Hancock AA (2006b) Differential CNS expression and functional activity of multiple human H_3 receptor isoforms. *Inflammation Research* V55:S38-S39.

Fang X, Yu SX, Lu Y, Bast RC, Jr., Woodgett JR and Mills GB (2000) Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc Natl Acad Sci U S A* 97:11960-11965.

Farooqui AA and Horrocks LA (2006) Phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly. *Neuroscientist* 12:245-260.

Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, Allen PB, Ouimet CC and Greengard P (2000) Spinophilin regulates the formation and function of dendritic spines. *Proc Natl Acad Sci U S A* 97:9287-9292.

Flanagan CA (2005) A GPCR that is not "DRY". *Mol Pharmacol* 68:1-3.

Fluhmann B, Zimmermann U, Muff R, Bilbe G, Fischer JA and Born W (1998) Parathyroid hormone responses of cyclic AMP-, serum- and phorbol ester-responsive reporter genes in osteoblast-like UMR-106 cells. *Mol Cell Endocrinol* 139:89-98.

Fox GB, Esbenshade TA, Pan JB, Radek RJ, Krueger KM, Yao BB, Browman KE, Buckley MJ, Ballard ME, Komater VA, Miner H, Zhang M, Faghih R, Rueter LE, Bitner RS, Drescher KU, Wetter J, Marsh K, Lemaire M, Porsolt RD, Bennani YL, Sullivan JP, Cowart MD, Decker MW and Hancock AA (2005) Pharmacological properties of ABT-239 [4-(2-{2-[(2R)-2-Methylpyrrolidinyl]ethyl}-benzofuran-5-yl)benzonitrile]: II. Neurophysiological characterization and broad preclinical efficacy in cognition and schizophrenia of a potent and selective histamine H3 receptor antagonist. *J Pharmacol Exp Ther* 313:176-190.

Fox GB, Pan JB, Esbenshade TA, Bennani YL, Black LA, Faghih R, Hancock AA and Decker MW (2002) Effects of histamine H(3) receptor ligands GT-2331 and ciproxifan in a repeated acquisition avoidance response in the spontaneously hypertensive rat pup. *Behav Brain Res* 131:151-161.

Frank M, Thumer L, Lohse MJ and Bunemann M (2005) G Protein activation without subunit dissociation depends on a G{alpha}(i)-specific region. *J Biol Chem* 280:24584-24590.

Fredriksson R, Lagerstrom MC, Lundin LG and Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 63:1256-1272.

Freston JW (2000) Management of peptic ulcers: emerging issues. *World J Surg* 24:250-255.

Fu H, Subramanian RR and Masters SC (2000) 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* 40:617-647.

Fukui H, Fujimoto K, Mizuguchi H, Sakamoto K, Horio Y, Takai S, Yamada K and Ito S (1994) Molecular cloning of the human histamine H_1 receptor gene. *Biochem Biophys Res Commun* 201:894-901.

Gallagher M and Yates SL (2003) Histamine H_3 receptor polynucleotides, in.

Gantz I, Munzert G, Tashiro T, Schaffer M, Wang L, DelValle J and Yamada T (1991a) Molecular cloning of the human histamine H_2 receptor. *Biochem Biophys Res Commun* 178:1386-1392.

Gantz I, Schaffer M, DelValle J, Logsdon C, Campbell V, Uhler M and Yamada T (1991b) Molecular cloning of a gene encoding the histamine H_2 receptor. *Proc Natl Acad Sci U S A* 88:5937.

Gantz I, Schaffer M, DelValle J, Logsdon C, Campbell V, Uhler M and Yamada T (1991c) Molecular cloning of a gene encoding the histamine H_2 receptor. *Proc Natl Acad Sci U S A* 88:429-433.

Gazi L, Nickolls SA and Strange PG (2003) Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. *Br J Pharmacol* 138:775-786.

Gbahou F, Rouleau A, Morisset S, Parmentier R, Crochet S, Lin JS, Ligneau X, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC and Arrang JM (2003) Protean agonism at histamine H_3 receptors in vitro and in vivo. *Proc Natl Acad Sci U S A* 100:11086-11091.

Giorgetti M, Bacciottini L, Bianchi L, Giovannini MG, Cecchi M and Blandina P (1997) GABAergic mechanism in histamine H3 receptor inhibition of K(+)-evoked release of acetylcholine from rat cortex in vivo. *Inflamm Res* 46 Suppl 1:S33-34.

Giovannini MG, Bartolini L, Bacciottini L, Greco L and Blandina P (1999) Effects of histamine H3 receptor agonists and antagonists on cognitive performance and scopolamine-induced amnesia. *Behav Brain Res* 104:147-155.

Giovannini MG, Efoudebe M, Passani MB, Baldi E, Bucherelli C, Giachi F, Corradetti R and Blandina P (2003) Improvement in fear memory by histamine-elicited ERK2 activation in hippocampal CA3 cells. *J Neurosci* 23:9016-9023.

Gomez-Ramirez J, Ortiz J and Blanco I (2002) Presynaptic H_3 autoreceptors modulate histamine synthesis through cAMP pathway. *Mol Pharmacol* 61:239-245.

Green JP, Prell GD, Khandelwal JK and Blandina P (1987) Aspects of histamine metabolism. *Agents Actions* 22:1-15.

Gutkind JS (2000) Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. *Sci STKE* 2000:RE1.

Haas H and Panula P (2003) The role of histamine and the tuberomamillary nucleus in the nervous system. *Nat Rev Neurosci* 4:121-130.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, Grinstein S and Lefkowitz RJ (1998) The beta2-adrenergic receptor interacts with the Na+/H+-exchanger

regulatory factor to control Na+/H+ exchange. *Nature* 392:626-630.

Hancock AA (2003) H_3 receptor antagonists/inverse agonists as anti-obesity agents. *Curr Opin Investig Drugs* 4:1190-1197.

Hancock AA (2006) The challenge of drug discovery of a GPCR target: analysis of preclinical pharmacology of histamine H_3 antagonists/inverse agonists. *Biochem Pharmacol* 71:1103-1113.

Hancock AA, Bennani YL, Bush EN, Esbenshade TA, Faghih R, Fox GB, Jacobson P, Knourek-Segel V, Krueger KM, Nuss ME, Pan JB, Shapiro R, Witte DG and Yao BB (2004a) Antiobesity effects of A-331440, a novel non-imidazole histamine H3 receptor antagonist. *Eur J Pharmacol* 487:183-197.

Hancock AA, Bitner RS, Krueger KM, Otte S, Nikkel AL, Fey TA, Bush EN, Dickinson RW, Shapiro R, Knourek-Segel V, Droz BA, Brune ME, Jacobson PB, Cowart MD and Esbenshade TA (2006) Distinctions and contradistinctions between antiobesity histamine H(3) receptor (H (3)R) antagonists compared to cognition-enhancing H (3) receptor antagonists. *Inflamm Res* 55 Suppl 1:S42-44.

Hancock AA and Brune ME (2005) Assessment of pharmacology and potential anti-obesity properties of H_3 receptor antagonists/inverse agonists. *Expert Opin Investig Drugs* 14:223-241.

Hancock AA, Diehl MS, Faghih R, Bush EN, Krueger KM, Krishna G, Miller TR, Wilcox DM, Nguyen P, Pratt JK, Cowart MD, Esbenshade TA and Jacobson PB (2004b) In vitro optimization of structure activity relationships of analogues of A-331440 combining radioligand receptor binding assays and micronucleus assays of potential antiobesity histamine H3 receptor antagonists. *Basic Clin Pharmacol Toxicol* 95:144-152.

Hancock AA, Diehl MS, Fey TA, Bush EN, Faghih R, Miller TR, Krueger KM, Pratt JK, Cowart MD, Dickinson RW, Shapiro R, Knourek-Segel VE, Droz BA, McDowell CA, Krishna G, Brune ME, Esbenshade TA and Jacobson PB (2005) Antiobesity evaluation of histamine H3 receptor (H3R) antagonist analogs of A-331440 with improved safety and efficacy. *Inflamm Res* 54 Suppl 1:S27-29.

Hancock AA, Esbenshade TA, Krueger KM and Yao BB (2003) Genetic and pharmacological aspects of histamine H3 receptor heterogeneity. *Life Sci* 73:3043-3072.

Hancock AA and Fox GB (2004) Perspectives on cognitive domains, H3 receptor ligands and neurological disease. *Expert Opin Investig Drugs* 13:1237-1248.

Harper EA, Shankley NP and Black JW (1999) Evidence that histamine homologues discriminate between H_3-receptors in guinea-pig cerebral cortex and ileum longitudinal muscle myenteric plexus. *Br J Pharmacol* 128:751-759.

Hepler JR (1999) Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* 20:376-382.

Heydorn A, Ward RJ, Jorgensen R, Rosenkilde MM, Frimurer TM, Milligan G and Kostenis E (2004) Identification of a novel site within G protein alpha subunits important for specificity of receptor-G protein interaction. *Mol Pharmacol* 66:250-259.

Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R and Haas HL (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* 49:253-278.

Hoffmann M, Ward RJ, Cavalli A, Carr IC and Milligan G (2001) Differential capacities of the RGS1, RGS16 and RGS-GAIP regulators of G protein signaling to enhance alpha2A-adrenoreceptor agonist-stimulated GTPase activity of G(o1)alpha. *J Neurochem* 78:797-806.

Hofstra CL, Desai PJ, Thurmond RL and Fung-Leung WP (2003) Histamine H_4 receptor mediates chemotaxis and calcium mobilization of mast cells. *J Pharmacol Exp Ther* 305:1212-1221.

Hu Y, Qiao L, Wang S, Rong SB, Meuillet EJ, Berggren M, Gallegos A, Powis G and Kozikowski AP (2000) 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J Med Chem* 43:3045-3051.

Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, Watanabe T, Urade Y and Hayaishi O (2001) Arousal effect of orexin A depends on activation of the histaminergic system. *Proc Natl Acad Sci U S A* 98:9965-9970.

Hubbell WL, Altenbach C, Hubbell CM and Khorana HG (2003) Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking. *Adv Protein Chem* 63:243-290.

Huston JP, Wagner U and Hasenohrl RU (1997) The tuberomammillary nucleus projections in the control of learning, memory and reinforcement processes: evidence for an inhibitory role. *Behav Brain Res* 83:97-105.

Ishikawa S and Sperelakis N (1987) A novel class (H3) of histamine receptors on perivascular nerve terminals. *Nature* 327:158-160.

Ishizuka T, Murakami M and Yamatodani A (2007) Involvement of central histaminergic systems in modafinil-induced but not methylphenidate-induced increases in locomotor activity in rats. *Eur J Pharmacol*.

Ishizuka T, Sakamoto Y, Sakurai T and Yamatodani A (2003) Modafinil increases

histamine release in the anterior hypothalamus of rats. *Neurosci Lett* 339:143-146.

Ishizuka T, Yamamoto Y and Yamatodani A (2002) The effect of orexin-A and -B on the histamine release in the anterior hypothalamus in rats. *Neurosci Lett* 323:93-96.

Itoh E, Fujimiya M and Inui A (1998) Thioperamide, a histamine H3 receptor antagonist, suppresses NPY-but not dynorphin A-induced feeding in rats. *Regul Pept* 75-76:373-376.

Jablonowski JA, Grice CA, Chai W, Dvorak CA, Venable JD, Kwok AK, Ly KS, Wei J, Baker SM, Desai PJ, Jiang W, Wilson SJ, Thurmond RL, Karlsson L, Edwards JP, Lovenberg TW and Carruthers NI (2003) The first potent and selective non-imidazole human histamine H_4 receptor antagonists. *J Med Chem* 46:3957-3960.

Jansen FP, Mochizuki T, Yamamoto Y, Timmerman H and Yamatodani A (1998) In vivo modulation of rat hypothalamic histamine release by the histamine H_3 receptor ligands, immepip and clobenpropit. Effects of intrahypothalamic and peripheral application. *Eur J Pharmacol* 362:149-155.

Jansen FP, Wu TS, Voss HP, Steinbusch HW, Vollinga RC, Rademaker B, Bast A and Timmerman H (1994) Characterization of the binding of the first selective radiolabelled histamine H_3-receptor antagonist, [^{125}I]-iodophenpropit, to rat brain. *Br J Pharmacol* 113:355-362.

Jean-Baptiste G, Yang Z and Greenwood MT (2006) Regulatory mechanisms involved in modulating RGS function. *Cell Mol Life Sci* 63:1969-1985.

Johnston CA and Siderovski DP (2007a) Receptor-mediated activation of heterotrimeric G-proteins: current structural insights. *Mol Pharmacol* 72:219-230.

Johnston CA and Siderovski DP (2007b) Structural basis for nucleotide exchange on G alpha i subunits and receptor coupling specificity. *Proc Natl Acad Sci U S A* 104:2001-2006.

Jones DH, Martin H, Madrazo J, Robinson KA, Nielsen P, Roseboom PH, Patel Y, Howell SA and Aitken A (1995) Expression and structural analysis of 14-3-3 proteins. *J Mol Biol* 245:375-384.

Jope RS and Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 29:95-102.

Kamei C, Okumura Y and Tasaka K (1993) Influence of histamine depletion on learning and memory recollection in rats. *Psychopharmacology (Berl)* 111:376-382.

Kamei C and Tasaka K (1993) Effect of histamine on memory retrieval in old rats. *Biol Pharm Bull* 16:128-132.

Karlstedt K, Ahman MJ, Anichtchik OV, Soinila S and Panula P (2003) Expression of the H3 receptor in the developing CNS and brown fat suggests novel roles for histamine. *Mol Cell Neurosci* 24:614-622.

Karmazyn M (1999) The role of the myocardial sodium-hydrogen exchanger in mediating ischemic and reperfusion injury. From amiloride to cariporide. *Ann N Y Acad Sci* 874:326-334.

Kay GG (2000) The effects of antihistamines on cognition and performance. *J Allergy Clin Immunol* 105:S622-627.

Kaytor MD and Orr HT (2002) The GSK3 beta signaling cascade and neurodegenerative disease. *Curr Opin Neurobiol* 12:275-278.

Kenakin T (1995) Pharmacological proteus? *Trends Pharmacol Sci* 16:256-258.

Kenakin T (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol* 65:2-11.

Kenakin T and Onaran O (2002) The ligand paradox between affinity and efficacy: can you be there and not make a difference? *Trends Pharmacol Sci* 23:275-280.

Kenakin TP (1989) Challenges for receptor theory as a tool for drug and drug receptor classification. *Trends Pharmacol Sci* 10:18-22.

Kenakin TP and Morgan PH (1989) Theoretical effects of single and multiple transducer receptor coupling proteins on estimates of the relative potency of agonists. *Mol Pharmacol* 35:214-222.

Kilduff TS and Peyron C (2000) The hypocretin/orexin ligand-receptor system: implications for sleep and sleep disorders. *Trends Neurosci* 23:359-365.

King BM (2006) The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. *Physiol Behav* 87:221-244.

Kitbunnadaj R, Zuiderveld OP, De Esch IJ, Vollinga RC, Bakker R, Lutz M, Spek AL, Cavoy E, Deltent MF, Menge WM, Timmerman H and Leurs R (2003) Synthesis and structure-activity relationships of conformationally constrained histamine H(3) receptor agonists. *J Med Chem* 46:5445-5457.

Kiyono S, Seo ML, Shibagaki M, Watanabe T, Maeyama K and Wada H (1985) Effects of alpha-fluoromethylhistidine on sleep-waking parameters in rats. *Physiol Behav* 34:615-617.

Klein S, Reuveni H and Levitzki A (2000) Signal transduction by a nondissociable heterotrimeric yeast G protein. *Proc Natl Acad Sci U S A* 97:3219-3223.

Kollonitsch J, Perkins LM, Patchett AA, Doldouras GA, Marburg S, Duggan DE, Maycock AL and Aster SD (1978) Selective inhibitors of biosynthesis of aminergic neurotransmitters. *Nature* 274:906-908.

Komater VA, Buckley MJ, Browman KE, Pan JB, Hancock AA, Decker MW and Fox GB (2005) Effects of histamine H3 receptor antagonists in two models of spatial learning. *Behav Brain Res* 159:295-300.

Kostenis E, Martini L, Ellis J, Waldhoer M, Heydorn A, Rosenkilde MM, Norregaard PK, Jorgensen R, Whistler JL and Milligan G (2005) A highly conserved glycine within linker I and the extreme C terminus of G protein alpha subunits interact cooperatively in switching G protein-coupled receptor-to-effector specificity. *J Pharmacol Exp Ther* 313:78-87.

Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* 103:21-80.

Krueger KM, Witte DG, Ireland-Denny L, Miller TR, Baranowski JL, Buckner S, Milicic I, Esbenshade TA and Hancock AA (2005) G protein-dependent pharmacology of histamine H_3 receptor ligands: evidence for heterogeneous active state receptor conformations. *J Pharmacol Exp Ther* 314:271-281.

Kuhn B, Schmid A, Harteneck C, Gudermann T and Schultz G (1996) G proteins of the Gq family couple the H_2 histamine receptor to phospholipase C. *Mol Endocrinol* 10:1697-1707.

Kurose H (2003) Galpha12 and Galpha13 as key regulatory mediator in signal transduction. *Life Sci* 74:155-161.

LaBella FS, Queen G, Glavin G, Durant G, Stein D and Brandes LJ (1992) H3 receptor antagonist, thioperamide, inhibits adrenal steroidogenesis and histamine binding to adrenocortical microsomes and binds to cytochrome P450. *Br J Pharmacol* 107:161-164.

Laitinen JT and Jokinen M (1998) Guanosine 5'-(gamma-[^{35}S]thio)triphosphate autoradiography allows selective detection of histamine H_3 receptor-dependent G protein activation in rat brain tissue sections. *J Neurochem* 71:808-816.

Lanctot PM, Leclerc PC, Clement M, Auger-Messier M, Escher E, Leduc R and Guillemette G (2005) Importance of N-glycosylation positioning for cell-surface expression, targeting, affinity and quality control of the human AT1 receptor. *Biochem J* 390:367-376.

Lecklin A, Etu-Seppala P, Stark H and Tuomisto L (1998) Effects of intracerebroventricularly infused histamine and selective H1, H2 and H3 agonists on food and water intake and urine flow in Wistar rats. *Brain Res* 793:279-288.

Lee JJ and Parsons ME (2000) Signaling mechanisms coupled to presynaptic A(1)- and H(3)-receptors in the inhibition of cholinergic contractile responses of the guinea pig ileum. *J Pharmacol Exp Ther* 295:607-613.

Leff P and Dougall IG (1993) Further concerns over Cheng-Prusoff analysis. *Trends Pharmacol Sci* 14:110-112.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* 14:303-307.

Lefkowitz RJ and Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. *Science* 308:512-517.

Leopoldt D, Harteneck C and Nurnberg B (1997) G proteins endogenously expressed in Sf 9 cells: interactions with mammalian histamine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 356:216-224.

Leurs R, Bakker RA, Timmerman H and de Esch IJ (2005) The histamine H3 receptor: from gene cloning to H3 receptor drugs. *Nat Rev Drug Discov* 4:107-120.

Leurs R, Blandina P, Tedford C and Timmerman H (1998) Therapeutic potential of histamine H_3 receptor agonists and antagonists. *Trends Pharmacol Sci* 19:177-183.

Leurs R, Hoffmann M, Wieland K and Timmerman H (2000) H_3 receptor gene is cloned at last. *Trends Pharmacol Sci* 21:11-12.

Leurs R, Kathmann M, Vollinga RC, Menge WM, Schlicker E and Timmerman H (1996) Histamine homologues discriminating between two functional H_3 receptor assays. Evidence for H_3 receptor heterogeneity? *J Pharmacol Exp Ther* 276:1009-1015.

Leurs R and Timmerman H (1992) The histamine H3-receptor: a target for developing new drugs. *Prog Drug Res* 39:127-165.

Leurs R, Vollinga RC and Timmerman H (1995) The medicinal chemistry and therapeutic potentials of ligands of the histamine H_3 receptor. *Prog Drug Res* 45:107-165.

Levi R and Smith NC (2000) Histamine H(3)-receptors: a new frontier in myocardial ischemia. *J Pharmacol Exp Ther* 292:825-830.

Levitzki A and Klein S (2002) G-protein subunit dissociation is not an integral part of G-protein action. *Chembiochem* 3:815-818.

Li X, Bijur GN and Jope RS (2002) Glycogen synthase kinase-3beta, mood stabilizers, and neuroprotection. *Bipolar Disord* 4:137-144.

Ligneau X, Perrin D, Landais L, Camelin JC, Calmels TP, Berrebi-Bertrand I, Lecomte JM, Parmentier R, Anaclet C, Lin JS, Bertaina-Anglade V, la Rochelle CD, d'Aniello F, Rouleau A, Gbahou F, Arrang JM, Ganellin CR, Stark H, Schunack W and Schwartz JC (2007) BF2.649 [1-{3-[3-(4-Chlorophenyl)propoxy]propyl}piperidine, hydrochloride], a nonimidazole inverse agonist/antagonist at the human histamine H3

receptor: Preclinical pharmacology. *J Pharmacol Exp Ther* 320:365-375.

Lim HD, Smits RA, Bakker RA, van Dam CM, de Esch IJ and Leurs R (2006) Discovery of S-(2-guanidylethyl)-isothiourea (VUF 8430) as a potent nonimidazole histamine H4 receptor agonist. *J Med Chem* 49:6650-6651.

Lim HD, van Rijn RM, Ling P, Bakker RA, Thurmond RL and Leurs R (2005) Evaluation of histamine H_1-, H_2-, and H_3-receptor ligands at the human histamine H_4 receptor: identification of 4-methylhistamine as the first potent and selective H_4 receptor agonist. *J Pharmacol Exp Ther* 314:1310-1321.

Lin JS (2000) Brain structures and mechanisms involved in the control of cortical activation and wakefulness, with emphasis on the posterior hypothalamus and histaminergic neurons. *Sleep Med Rev* 4:471-503.

Lin JS, Sakai K and Jouvet M (1988) Evidence for histaminergic arousal mechanisms in the hypothalamus of cat. *Neuropharmacology* 27:111-122.

Lin JS, Sakai K, Vanni-Mercier G, Arrang JM, Garbarg M, Schwartz JC and Jouvet M (1990) Involvement of histaminergic neurons in arousal mechanisms demonstrated with H3-receptor ligands in the cat. *Brain Res* 523:325-330.

Lintunen M, Sallmen T, Karlstedt K and Panula P (2005) Transient changes in the limbic histaminergic system after systemic kainic acid-induced seizures. *Neurobiol Dis* 20:155-169.

Liu C, Ma X, Jiang X, Wilson SJ, Hofstra CL, Blevitt J, Pyati J, Li X, Chai W, Carruthers N and Lovenberg TW (2001a) Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in bone marrow. *Mol Pharmacol* 59:420-426.

Liu C, Wilson SJ, Kuei C and Lovenberg TW (2001b) Comparison of human, mouse, rat, and guinea pig histamine H_4 receptors reveals substantial pharmacological species variation. *J Pharmacol Exp Ther* 299:121-130.

Liu W and Northup JK (1998) The helical domain of a G protein alpha subunit is a regulator of its effector. *Proc Natl Acad Sci U S A* 95:12878-12883.

Lovenberg TW, Pyati J, Chang H, Wilson SJ and Erlander MG (2000) Cloning of rat histamine H(3) receptor reveals distinct species pharmacological profiles. *J Pharmacol Exp Ther* 293:771-778.

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

Lozada A, Munyao N, Sallmen T, Lintunen M, Leurs R, Lindsberg PJ and Panula P (2005) Postischemic regulation of central histamine receptors. *Neuroscience* 136:371-379.

Lu ZL, Curtis CA, Jones PG, Pavia J and Hulme EC (1997) The role of the aspartate-arginine-tyrosine triad in the m1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. *Mol Pharmacol* 51:234-241.

Luttrell DK and Luttrell LM (2003) Signaling in time and space: G protein-coupled receptors and mitogen-activated protein kinases. *Assay Drug Dev Technol* 1:327-338.

Machidori H, Sakata T, Yoshimatsu H, Ookuma K, Fujimoto K, Kurokawa M, Yamatodani A and Wada H (1992) Zucker obese rats: defect in brain histamine control of feeding. *Brain Res* 590:180-186.

Mackay D (1990) Agonist potency and apparent affinity: interpretation using classical and steady-state ternary-complex models. *Trends Pharmacol Sci* 11:17-22.

MacMillan LB, Bass MA, Cheng N, Howard EF, Tamura M, Strack S, Wadzinski BE and Colbran RJ (1999) Brain actin-associated protein phosphatase 1 holoenzymes containing spinophilin, neurabin, and selected catalytic subunit isoforms. *J Biol Chem* 274:35845-35854.

Malinowska B, Godlewski G and Schlicker E (1998) Histamine H3 receptors--general characterization and their function in the cardiovascular system. *J Physiol Pharmacol* 49:191-211.

Malmlof K, Golozoubova V, Peschke B, Wulff BS, Refsgaard HH, Johansen PB, Cremers T and Rimvall K (2006) Increase of neuronal histamine in obese rats is associated with decreases in body weight and plasma triglycerides. *Obesity (Silver Spring)* 14:2154-2162.

Malmlof K, Hastrup S, Wulff BS, Hansen BC, Peschke B, Jeppesen CB, Hohlweg R and Rimvall K (2007) Antagonistic targeting of the histamine H3 receptor decreases caloric intake in higher mammalian species. *Biochem Pharmacol* 73:1237-1242.

Malmlof K, Zaragoza F, Golozoubova V, Refsgaard HH, Cremers T, Raun K, Wulff BS, Johansen PB, Westerink B and Rimvall K (2005) Influence of a selective histamine H3 receptor antagonist on hypothalamic neural activity, food intake and body weight. *Int J Obes (Lond)* 29:1402-1412.

Martinez-Mir MI, Pollard H, Moreau J, Arrang JM, Ruat M, Traiffort E, Schwartz JC and Palacios JM (1990) Three histamine receptors (H1, H2 and H3) visualized in the brain of human and non-human primates. *Brain Res* 526:322-327.

McLeod RL, Aslanian R, del Prado M, Duffy R, Egan RW, Kreutner W, McQuade R and Hey JA

(1998) Sch 50971, an orally active histamine H3 receptor agonist, inhibits central neurogenic vascular inflammation and produces sedation in the guinea pig. *J Pharmacol Exp Ther* 287:43-50.

Medhurst AD, Atkins AR, Beresford IJ, Brackenborough K, Briggs MA, Calver AR, Cilia J, Cluderay JE, Crook B, Davis JB, Davis RK, Davis RP, Dawson LA, Foley AG, Gartlon J, Gonzalez MI, Heslop T, Hirst WD, Jennings C, Jones DN, Lacroix LP, Martyn A, Ociepka S, Ray A, Regan CM, Roberts JC, Schogger J, Southam E, Stean TO, Trail BK, Upton N, Wadsworth G, Wald JA, White T, Witherington J, Woolley ML, Worby A and Wilson DM (2007) GSK189254, a novel H3 receptor antagonist that binds to histamine H3 receptors in Alzheimer's disease brain and improves cognitive performance in preclinical models. *J Pharmacol Exp Ther* 321:1032-1045.

Mercer LP, Kelley DS, Humphries LL and Dunn JD (1994) Manipulation of central nervous system histamine or histaminergic receptors (H1) affects food intake in rats. *J Nutr* 124:1029-1036.

Michineau S, Alhenc-Gelas F and Rajerison RM (2006) Human bradykinin B2 receptor sialylation and N-glycosylation participate with disulfide bonding in surface receptor dimerization. *Biochemistry* 45:2699-2707.

Milligan G (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem J* 255:1-13.

Milligan G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* 64:1271-1276.

Milligan G, Bond RA and Lee M (1995) Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* 16:10-13.

Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* 147 Suppl 1:S46-55.

Missale C, Nash SR, Robinson SW, Jaber M and Caron MG (1998) Dopamine receptors: from structure to function. *Physiol Rev* 78:189-225.

Mittal V and Linder ME (2004) The RGS14 GoLoco domain discriminates among Galphai isoforms. *J Biol Chem* 279:46772-46778.

Miyazaki S, Imaizumi M and Onodera K (1995) Effects of thioperamide on the cholinergic system and the step-through passive avoidance test in mice. *Methods Find Exp Clin Pharmacol* 17:653-658.

Miyazaki S, Onodera K, Imaizumi M and Timmerman H (1997) Effects of clobenpropit (VUF-9153), a histamine H3-receptor antagonist, on learning and memory, and on cholinergic and monoaminergic systems in mice. *Life Sci* 61:355-361.

Moguilevsky N, Varsalona F, Noyer M, Gillard M, Guillaume JP, Garcia L, Szpirer C, Szpirer J and Bollen A (1994) Stable expression of human H_1-histamine-receptor cDNA in Chinese hamster ovary cells. Pharmacological characterisation of the protein, tissue distribution of messenger RNA and chromosomal localisation of the gene. *Eur J Biochem* 224:489-495.

Molina-Hernandez A, Nunez A, Sierra JJ and Arias-Montano JA (2001) Histamine H3 receptor activation inhibits glutamate release from rat striatal synaptosomes. *Neuropharmacology* 41:928-934.

Monczor F, Fernandez N, Legnazzi BL, Riveiro ME, Baldi A, Shayo C and Davio C (2003) Tiotidine, a histamine H_2 receptor inverse agonist that binds with high affinity to an inactive G-protein-coupled form of the receptor. Experimental support for the cubic ternary complex model. *Mol Pharmacol* 64:512-520.

Monti JM, Jantos H, Boussard M, Altier H, Orellana C and Olivera S (1991) Effects of selective activation or blockade of the histamine H3 receptor on sleep and wakefulness. *Eur J Pharmacol* 205:283-287.

Monti JM, Jantos H, Ponzoni A and Monti D (1996) Sleep and waking during acute histamine H3 agonist BP 2.94 or H3 antagonist carboperamide (MR 16155) administration in rats. *Neuropsychopharmacology* 15:31-35.

Moreno-Delgado D, Torrent A, Gomez-Ramirez J, de Esch I, Blanco I and Ortiz J (2006a) Constitutive activity of H3 autoreceptors modulates histamine synthesis in rat brain through the cAMP/PKA pathway. *Neuropharmacology* 51:517-523.

Moreno-Delgado D, Torrent A, Gomez-Ramirez J, de Esch I, Blanco I and Ortiz J (2006b) Constitutive activity of H(3) autoreceptors modulates histamine synthesis in rat brain through the cAMP/PKA pathway. *Neuropharmacology*.

Morisset S, Rouleau A, Ligneau X, Gbahou F, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC and Arrang JM (2000) High constitutive activity of native H_3 receptors regulates histamine neurons in brain. *Nature* 408:860-864.

Morisset S, Sasse A, Gbahou F, Heron A, Ligneau X, Tardivel-Lacombe J, Schwartz JC and Arrang JM (2001) The rat H_3 receptor: gene organization and multiple isoforms. *Biochem Biophys Res Commun* 280:75-80.

Morse KL, Behan J, Laz TM, West RE, Jr., Greenfeder SA, Anthes JC, Umland S, Wan Y, Hipkin RW, Gonsiorek W, Shin N, Gustafson EL, Qiao X, Wang S, Hedrick JA, Greene J, Bayne M and Monsma FJ, Jr. (2001) Cloning and characterization of a novel human histamine receptor. *J Pharmacol Exp Ther* 296:1058-1066.

Murga C, Laguinge L, Wetzker R, Cuadrado A and Gutkind JS (1998) Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. *J Biol Chem* 273:19080-19085.

Muslin AJ, Tanner JW, Allen PM and Shaw AS (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* 84:889-897.

Nakamura T, Itadani H, Hidaka Y, Ohta M and Tanaka K (2000) Molecular cloning and characterization of a new human histamine receptor, HH4R. *Biochem Biophys Res Commun* 279:615-620.

Nakayama T, Kato Y, Hieshima K, Nagakubo D, Kunori Y, Fujisawa T and Yoshie O (2004) Liver-expressed chemokine/CC chemokine ligand 16 attracts eosinophils by interacting with histamine H_4 receptor. *J Immunol* 173:2078-2083.

Newman-Tancredi A, Conte C, Chaput C, Verriele L and Millan MJ (1997) Agonist and inverse agonist efficacy at human recombinant serotonin 5-HT1A receptors as a function of receptor:G-protein stoichiometry. *Neuropharmacology* 36:451-459.

Nguyen T, Shapiro DA, George SR, Setola V, Lee DK, Cheng R, Rauser L, Lee SP, Lynch KR, Roth BL and O'Dowd BF (2001) Discovery of a novel member of the histamine receptor family. *Mol Pharmacol* 59:427-433.

Niu J, Scheschonka A, Druey KM, Davis A, Reed E, Kolenko V, Bodnar R, Voyno-Yasenetskaya T, Du X, Kehrl J and Dulin NO (2002) RGS3 interacts with 14-3-3 via the N-terminal region distinct from the RGS (regulator of G-protein signalling) domain. *Biochem J* 365:677-684.

Nordberg A (1992) Neuroreceptor changes in Alzheimer disease. *Cerebrovasc Brain Metab Rev* 4:303-328.

Oda T, Morikawa N, Saito Y, Masuho Y and Matsumoto S (2000) Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J Biol Chem* 275:36781-36786.

Oike M, Kitamura K and Kuriyama H (1992) Histamine H3-receptor activation augments voltage-dependent Ca2+ current via GTP hydrolysis in rabbit saphenous artery. *J Physiol* 448:133-152.

Okamura N, Yanai K, Higuchi M, Sakai J, Iwata R, Ido T, Sasaki H, Watanabe T and Itoh M (2000) Functional neuroimaging of cognition impaired by a classical antihistamine, d-chlorpheniramine. *Br J Pharmacol* 129:115-123.

Oldham WM and Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9:60-71.

Onodera K, Yamatodani A, Watanabe T and Wada H (1994) Neuropharmacology of the histaminergic neuron system in the brain and its relationship with behavioral disorders. *Prog Neurobiol* 42:685-702.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745.

Panula P, Rinne J, Kuokkanen K, Eriksson KS, Sallmen T, Kalimo H and Relja M (1998) Neuronal histamine deficit in Alzheimer's disease. *Neuroscience* 82:993-997.

Panula P, Yang HY and Costa E (1984) Histamine-containing neurons in the rat hypothalamus. *Proc Natl Acad Sci U S A* 81:2572-2576.

Pao CS and Benovic JL (2005) Structure/function analysis of alpha2A-adrenergic receptor interaction with G protein-coupled receptor kinase 2. *J Biol Chem* 280:11052-11058.

Parmentier R, Anaclet C, Guhennec C, Brousseau E, Bricout D, Giboulot T, Bozyczko-Coyne D, Spiegel K, Ohtsu H, Williams M and Lin JS (2007) The brain H3-receptor as a novel therapeutic target for vigilance and sleep-wake disorders. *Biochem Pharmacol* 73:1157-1171.

Parmentier R, Ohtsu H, Djebbara-Hannas Z, Valatx JL, Watanabe T and Lin JS (2002) Anatomical, physiological, and pharmacological characteristics of histidine decarboxylase knock-out mice: evidence for the role of brain histamine in behavioral and sleep-wake control. *J Neurosci* 22:7695-7711.

Passani MB, Cangioli I, Baldi E, Bucherelli C, Mannaioni PF and Blandina P (2001) Histamine H3 receptor-mediated impairment of contextual fear conditioning and in-vivo inhibition of cholinergic transmission in the rat basolateral amygdala. *Eur J Neurosci* 14:1522-1532.

Passani MB, Lin JS, Hancock A, Crochet S and Blandina P (2004) The histamine H3 receptor as a novel therapeutic target for cognitive and sleep disorders. *Trends Pharmacol Sci* 25:618-625.

Pauwels PJ, Rauly I and Wurch T (2003) Dissimilar pharmacological responses by a new series of imidazoline derivatives at precoupled and ligand-activated alpha 2A-adrenoceptor states: evidence for effector pathway-dependent differential antagonism. *J Pharmacol Exp Ther* 305:1015-1023.

Penston JG (1996) Review article: clinical aspects of Helicobacter pylori eradication therapy in peptic ulcer disease. *Aliment Pharmacol Ther* 10:469-486.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG and Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18:9996-10015.

Pfleger KD and Eidne KA (2005) Monitoring the formation of dynamic G-protein-coupled receptor-protein complexes in living cells. *Biochem J* 385:625-637.

Phillis JW (2005) Acetylcholine release from the central nervous system: a 50-year retrospective. *Crit Rev Neurobiol* 17:161-217.

Poli E, Pozzoli C, Coruzzi G and Bertaccini G (1993) Histamine H_3-receptor-induced inhibition of duodenal cholinergic transmission is independent of intracellular cyclic AMP and GMP. *Gen Pharmacol* 24:1273-1278.

Poli E, Pozzoli C, Coruzzi G and Bertaccini G (1994) Signal transducing mechanisms coupled to histamine H3 receptors and alpha-2 adrenoceptors in the guinea pig duodenum: possible involvement of N-type Ca++ channels. *J Pharmacol Exp Ther* 270:788-794.

Pozuelo Rubio M, Geraghty KM, Wong BH, Wood NT, Campbell DG, Morrice N and Mackintosh C (2004) 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. *Biochem J* 379:395-408.

Prast H, Argyriou A and Philippu A (1996) Histaminergic neurons facilitate social memory in rats. *Brain Res* 734:316-318.

Premont RT and Gainetdinov RR (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol* 69:511-534.

Prezeau L, Richman JG, Edwards SW and Limbird LE (1999) The zeta isoform of 14-3-3 proteins interacts with the third intracellular loop of different alpha2-adrenergic receptor subtypes. *J Biol Chem* 274:13462-13469.

Raible DG, Lenahan T, Fayvilevich Y, Kosinski R and Schulman ES (1994) Pharmacologic characterization of a novel histamine receptor on human eosinophils. *Am J Respir Crit Care Med* 149:1506-1511.

Rands E, Candelore MR, Cheung AH, Hill WS, Strader CD and Dixon RA (1990) Mutational analysis of beta-adrenergic receptor glycosylation. *J Biol Chem* 265:10759-10764.

Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, Ping P and McLeish KR (2001) p38 Kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. *J Biol Chem* 276:3517-3523.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI and Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450:383-387.

Richman JG, Brady AE, Wang Q, Hensel JL, Colbran RJ and Limbird LE (2001) Agonist-regulated Interaction between alpha2-adrenergic receptors and spinophilin. *J Biol Chem* 276:15003-15008.

Rickle A, Bogdanovic N, Volkman I, Winblad B, Ravid R and Cowburn RF (2004) Akt activity in Alzheimer's disease and other neurodegenerative disorders. *Neuroreport* 15:955-959.

Riobo NA and Manning DR (2005) Receptors coupled to heterotrimeric G proteins of the G12 family. *Trends Pharmacol Sci* 26:146-154.

Rodbell M, Krans HM, Pohl SL and Birnbaumer L (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. IV. Effects of guanylnucleotides on binding of 125I-glucagon. *J Biol Chem* 246:1872-1876.

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC and Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* 318:1266-1273.

Rouleau A, Heron A, Cochois V, Pillot C, Schwartz JC and Arrang JM (2004) Cloning and expression of the mouse histamine H_3 receptor: evidence for multiple isoforms. *J Neurochem* 90:1331-1338.

Rovati GE, Capra V and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* 71:959-964.

Sable CL, Filippa N, Hemmings B and Van Obberghen E (1997) cAMP stimulates protein kinase B in a Wortmannin-insensitive manner. *FEBS Lett* 409:253-257.

Sadee W, Wang D and Bilsky EJ (2005) Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. *Life Sci* 76:1427-1437.

Sakata T (1995) Histamine receptor and its regulation of energy metabolism. *Obes Res* 3 Suppl 4:541S-548S.

Sakata T, Ookuma K, Fujimoto K, Fukagawa K and Yoshimatsu H (1991) Histaminergic control of energy balance in rats. *Brain Res Bull* 27:371-375.

Sakata T, Yoshimatsu H and Kurokawa M (1997) Hypothalamic neuronal histamine: implications of its homeostatic control of energy metabolism. *Nutrition* 13:403-411.

Salamone JD, McLaughlin PJ, Sink K, Makriyannis A and Parker LA (2007) Cannabinoid CB1 receptor inverse agonists and neutral antagonists: effects on food intake, food-

reinforced behavior and food aversions. *Physiol Behav* 91:383-388.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* 268:4625-4636.

Sanchez-Lemus E and Arias-Montano JA (2004) Histamine H_3 receptor activation inhibits dopamine D_1 receptor-induced cAMP accumulation in rat striatal slices. *Neurosci Lett* 364:179-184.

Satoh A, Nakanishi H, Obaishi H, Wada M, Takahashi K, Satoh K, Hirao K, Nishioka H, Hata Y, Mizoguchi A and Takai Y (1998) Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell-cell adhesion sites. *J Biol Chem* 273:3470-3475.

Scheer A, Costa T, Fanelli F, De Benedetti PG, Mhaouty-Kodja S, Abuin L, Nenniger-Tosato M and Cotecchia S (2000) Mutational analysis of the highly conserved arginine within the Glu/Asp-Arg-Tyr motif of the alpha(1b)-adrenergic receptor: effects on receptor isomerization and activation. *Mol Pharmacol* 57:219-231.

Scheer A, Fanelli F, Costa T, De Benedetti PG and Cotecchia S (1997) The activation process of the alpha1B-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate. *Proc Natl Acad Sci U S A* 94:808-813.

Schlicker E, Behling A, Lummen G and Gothert M (1992) Histamine H_3A receptor-mediated inhibition of noradrenaline release in the mouse brain cortex. *Naunyn Schmiedebergs Arch Pharmacol* 345:489-493.

Schlicker E, Betz R and Gothert M (1988) Histamine H_3 receptor-mediated inhibition of serotonin release in the rat brain cortex. *Naunyn Schmiedebergs Arch Pharmacol* 337:588-590.

Schlicker E, Fink K, Detzner M and Gothert M (1993) Histamine inhibits dopamine release in the mouse striatum via presynaptic H_3 receptors. *J Neural Transm Gen Sect* 93:1-10.

Schlicker E, Fink K, Hinterthaner M and Gothert M (1989) Inhibition of noradrenaline release in the rat brain cortex via presynaptic H_3 receptors. *Naunyn Schmiedebergs Arch Pharmacol* 340:633-638.

Schlicker E, Kathmann M, Detzner M, Exner HJ and Gothert M (1994a) H_3 receptor-mediated inhibition of noradrenaline release: an investigation into the involvement of Ca^{2+} and K^+ ions, G protein and adenylate cyclase. *Naunyn Schmiedebergs Arch Pharmacol* 350:34-41.

Schlicker E, Malinowska B, Kathmann M and Gothert M (1994b) Modulation of neurotransmitter release via histamine H3 heteroreceptors. *Fundam Clin Pharmacol* 8:128-137.

Schneider C, Risser D, Kirchner L, Kitzmuller E, Cairns N, Prast H, Singewald N and Lubec G (1997) Similar deficits of central histaminergic system in patients with Down syndrome and Alzheimer disease. *Neurosci Lett* 222:183-186.

Schwartz JC, Arrang JM, Garbarg M and Pollard H (1990) Plenary lecture. A third histamine receptor subtype: characterisation, localisation and functions of the H3-receptor. *Agents Actions* 30:13-23.

Schwartz JC, Arrang JM, Garbarg M, Pollard H and Ruat M (1991) Histaminergic transmission in the mammalian brain. *Physiol Rev* 71:1-51.

Schwartz JC, Morisset S, Rouleau A, Ligneau X, Gbahou F, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR and Arrang JM (2003) Therapeutic implications of constitutive activity of receptors: the example of the histamine H3 receptor. *J Neural Transm Suppl*:1-16.

Segarra J, Balenci L, Drenth T, Maina F and Lamballe F (2006) Combined signaling through ERK, PI3K/AKT, and RAC1/p38 is required for met-triggered cortical neuron migration. *J Biol Chem* 281:4771-4778.

Seifert R and Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch Pharmacol* 366:381-416.

Servant G, Dudley DT, Escher E and Guillemette G (1996) Analysis of the role of N-glycosylation in cell-surface expression and binding properties of angiotensin II type-2 receptor of rat pheochromocytoma cells. *Biochem J* 313 (Pt 1):297-304.

Service RF (2004) Surviving the blockbuster syndrome. *Science* 303:1796-1799.

Seyedi N, Mackins CJ, Machida T, Reid AC, Silver RB and Levi R (2005) Histamine H_3-receptor-induced attenuation of norepinephrine exocytosis: a decreased protein kinase a activity mediates a reduction in intracellular calcium. *J Pharmacol Exp Ther* 312:272-280.

Shenoy SK and Lefkowitz RJ (2005) Seven-transmembrane receptor signaling through beta-arrestin. *Sci STKE* 2005:cm10.

Shenton FC, Hann V and Chazot PL (2005) Evidence for native and cloned H_3 histamine receptor higher oligomers. *Inflamm Res* 54 Suppl 1:S48-49.

Shibata T, Suzuki C, Ohnishi J, Murakami K and Miyazaki H (1996) Identification of regions in the human angiotensin II receptor type 1 responsible for Gi and Gq coupling by mutagenesis study. *Biochem Biophys Res Commun* 218:383-389.

Shiraishi-Yamaguchi Y and Furuichi T (2007) The Homer family proteins. *Genome Biol* 8:206.

Shu FJ, Ramineni S, Amyot W and Hepler JR (2007) Selective interactions between Gi alpha1 and Gi alpha3 and the GoLoco/GPR domain of RGS14 influence its dynamic subcellular localization. *Cell Signal* 19:163-176.

Siffert W, Jakobs KH and Akkerman JW (1990) Sodium fluoride prevents receptor- and protein kinase C-mediated activation of the human platelet Na+/H+ exchanger without inhibiting its basic pHi-regulating activity. *J Biol Chem* 265:15441-15448.

Silver RB, Mackins CJ, Smith NC, Koritchneva IL, Lefkowitz K, Lovenberg TW and Levi R (2001) Coupling of histamine H_3 receptors to neuronal Na^+/H^+ exchange: a novel protective mechanism in myocardial ischemia. *Proc Natl Acad Sci U S A* 98:2855-2859.

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

Skiba NP, Yang CS, Huang T, Bae H and Hamm HE (1999) The alpha-helical domain of Galphat determines specific interaction with regulator of G protein signaling 9. *J Biol Chem* 274:8770-8778.

Smit MJ, Leurs R, Alewijnse AE, Blauw J, Van Nieuw Amerongen GP, Van De Vrede Y, Roovers E and Timmerman H (1996) Inverse agonism of histamine H2 antagonist accounts for upregulation of spontaneously active histamine H2 receptors. *Proc Natl Acad Sci U S A* 93:6802-6807.

Smit MJ, Vischer HF, Bakker RA, Jongejan A, Timmerman H, Pardo L and Leurs R (2007) Pharmacogenomic and structural analysis of constitutive g protein-coupled receptor activity. *Annu Rev Pharmacol Toxicol* 47:53-87.

Smith CP, Hunter AJ and Bennett GW (1994) Effects of (R)-alpha-methylhistamine and scopolamine on spatial learning in the rat assessed using a water maze. *Psychopharmacology (Berl)* 114:651-656.

Smith FD, Oxford GS and Milgram SL (1999) Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J Biol Chem* 274:19894-19900.

Smith JS, Imagawa T, Ma J, Fill M, Campbell KP and Coronado R (1988) Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J Gen Physiol* 92:1-26.

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA and Bleasdale JE (1990) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J Pharmacol Exp Ther* 253:688-697.

Stambolic V and Woodgett JR (1994) Mitogen inactivation of glycogen synthase kinase-3 beta in intact cells via serine 9 phosphorylation. *Biochem J* 303 (Pt 3):701-704.

Stark H, Kathmann M, Schlicker E, Schunack W, Schlegel B and Sippl W (2004) Medicinal chemical and pharmacological aspects of imidazole-containing histamine H3 receptor antagonists. *Mini Rev Med Chem* 4:965-977.

Stark H, Purand K, Ligneau X, Rouleau A, Arrang JM, Garbarg M, Schwartz JC and Schunack W (1996) Novel carbamates as potent histamine H_3 receptor antagonists with high in vitro and oral in vivo activity. *J Med Chem* 39:1157-1163.

Sternweis PC, Northup JK, Smigel MD and Gilman AG (1981) The regulatory component of adenylate cyclase. Purification and properties. *J Biol Chem* 256:11517-11526.

Storga D, Vrecko K, Birkmayer JG and Reibnegger G (1996) Monoaminergic neurotransmitters, their precursors and metabolites in brains of Alzheimer patients. *Neurosci Lett* 203:29-32.

Sumner BE, Cruise LA, Slattery DA, Hill DR, Shahid M and Henry B (2004) Testing the validity of c-fos expression profiling to aid the therapeutic classification of psychoactive drugs. *Psychopharmacology (Berl)* 171:306-321.

Takahashi K, Suwa H, Ishikawa T and Kotani H (2002) Targeted disruption of H3 receptors results in changes in brain histamine tone leading to an obese phenotype. *J Clin Invest* 110:1791-1799.

Takeshita Y, Watanabe T, Sakata T, Munakata M, Ishibashi H and Akaike N (1998) Histamine modulates high-voltage-activated calcium channels in neurons dissociated from the rat tuberomammillary nucleus. *Neuroscience* 87:797-805.

Tardivel-Lacombe J, Morisset S, Gbahou F, Schwartz JC and Arrang JM (2001) Chromosomal mapping and organization of the human histamine H_3 receptor gene. *Neuroreport* 12:321-324.

Tardivel-Lacombe J, Rouleau A, Heron A, Morisset S, Pillot C, Cochois V, Schwartz JC and Arrang JM (2000) Cloning and cerebral expression of the guinea pig histamine H_3 receptor: evidence for two isoforms. *Neuroreport* 11:755-759.

Terzioglu N, van Rijn RM, Bakker RA, De Esch IJ and Leurs R (2004) Synthesis and structure-activity relationships of indole and benzimidazole piperazines as histamine H(4) receptor antagonists. *Bioorg Med Chem Lett* 14:5251-5256.

Thiels E and Klann E (2001) Extracellular signal-regulated kinase, synaptic plasticity, and memory. *Rev Neurosci* 12:327-345.

Thomas GM and Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* 5:173-183.

Thomas SM and Brugge JS (1997) Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 13:513-609.

Tokita S, Takahashi K and Kotani H (2006) Recent advances in molecular pharmacology of the histamine systems: physiology and pharmacology of histamine H3 receptor: roles in feeding regulation and therapeutic potential for metabolic disorders. *J Pharmacol Sci* 101:12-18.

Torrent A, Moreno-Delgado D, Gomez-Ramirez J, Rodriguez-Agudo D, Rodriguez-Caso C, Sanchez-Jimenez F, Blanco I and Ortiz J (2005) H3 autoreceptors modulate histamine synthesis through calcium/calmodulin- and cAMP-dependent protein kinase pathways. *Mol Pharmacol* 67:195-203.

Toyota H, Dugovic C, Koehl M, Laposky AD, Weber C, Ngo K, Wu Y, Lee DH, Yanai K, Sakurai E, Watanabe T, Liu C, Chen J, Barbier AJ, Turek FW, Fung-Leung WP and Lovenberg TW (2002) Behavioral characterization of mice lacking histamine H(3) receptors. *Mol Pharmacol* 62:389-397.

Tsien RY (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19:2396-2404.

Tsui P (2001a) Human histamine H_3 gene variant-2, in.

Tsui P (2001b) Human histamine H_3 gene variant-3, in.

Tsukada M, Prokscha A and Eichele G (2006) Neurabin II mediates doublecortin-dephosphorylation on actin filaments. *Biochem Biophys Res Commun* 343:839-847.

Tsukada M, Prokscha A, Ungewickell E and Eichele G (2005) Doublecortin association with actin filaments is regulated by neurabin II. *J Biol Chem* 280:11361-11368.

Tuomisto L, Yamatodani A, Jolkkonen J, Sainio EL and Airaksinen MM (1994) Inhibition of brain histamine synthesis increases food intake and attenuates vasopressin response to salt loading in rats. *Methods Find Exp Clin Pharmacol* 16:355-359.

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

van der Goot H, Schepers MJP, Sterk GJ and Timmerman H (1992) Isothiourea analogues of histamine as potent agonists or antagonists of the histamine H3-receptor. *European Journal of Medicinal Chemistry* 27:511-517.

van Willigen G, Nieuwland R, Nurnberg B, Gorter G and Akkerman JW (2000) Negative regulation of the platelet Na+/H+ exchanger by trimeric G-proteins. *Eur J Biochem* 267:7102-7108.

Violin JD and Lefkowitz RJ (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* 28:416-422.

Visiers I, Ballesteros JA and Weinstein H (2002) Three-dimensional representations of G protein-coupled receptor structures and mechanisms. *Methods Enzymol* 343:329-371.

Vollinga RC, Menge WM, Leurs R and Timmerman H (1995a) Homologs of histamine as histamine H_3 receptor antagonists: a new potent and selective H_3 antagonist, 4(5)-(5-aminopentyl)-1H-imidazole. *J Med Chem* 38:266-271.

Vollinga RC, Menge WM, Leurs R and Timmerman H (1995b) New analogs of burimamide as potent and selective histamine H_3 receptor antagonists: the effect of chain length variation of the alkyl spacer and modifications of the N-thiourea substituent. *J Med Chem* 38:2244-2250.

Waltereit R and Weller M (2003) Signaling from cAMP/PKA to MAPK and synaptic plasticity. *Mol Neurobiol* 27:99-106.

Wang H, Silva NL, Lucchesi PA, Haworth R, Wang K, Michalak M, Pelech S and Fliegel L (1997) Phosphorylation and regulation of the Na+/H+ exchanger through mitogen-activated protein kinase. *Biochemistry* 36:9151-9158.

Wang Q and Limbird LE (2002) Regulated interactions of the alpha 2A adrenergic receptor with spinophilin, 14-3-3zeta, and arrestin 3. *J Biol Chem* 277:50589-50596.

Wang Q and Limbird LE (2007) Regulation of alpha2AR trafficking and signaling by interacting proteins. *Biochem Pharmacol* 73:1135-1145.

Wang Q, Zhao J, Brady AE, Feng J, Allen PB, Lefkowitz RJ, Greengard P and Limbird LE (2004) Spinophilin blocks arrestin actions in vitro and in vivo at G protein-coupled receptors. *Science* 304:1940-1944.

Wang X, Zeng W, Kim MS, Allen PB, Greengard P and Muallem S (2007) Spinophilin/neurabin reciprocally regulate signaling intensity by G protein-coupled receptors. *Embo J* 26:2768-2776.

Wang X, Zeng W, Soyombo AA, Tang W, Ross EM, Barnes AP, Milgram SL, Penninger JM, Allen PB, Greengard P and Muallem S (2005) Spinophilin regulates Ca2+ signalling by binding the N-terminal domain of RGS2 and the third intracellular loop of G-protein-coupled receptors. *Nat Cell Biol* 7:405-411.

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG and Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* 454:486-491.

Watanabe T, Taguchi Y, Shiosaka S, Tanaka J, Kubota H, Terano Y, Tohyama M and Wada H (1984) Distribution of the histaminergic neuron system in the central nervous system of rats; a fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. *Brain Res* 295:13-25.

Weinman EJ, Hall RA, Friedman PA, Liu-Chen LY and Shenolikar S (2006) The association of NHERF adaptor proteins with g protein-coupled receptors and receptor tyrosine kinases. *Annu Rev Physiol* 68:491-505.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996a) The Cubic Ternary Complex Receptor-Occupancy Model I. Model Description. *Journal of Theoretical Biology* 178:151-167.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996b) The Cubic Ternary Complex Receptor-Occupancy Model II. Understanding Apparent Affinity. *Journal of Theoretical Biology* 178:169-182.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996c) The Cubic Ternary Complex Receptor-Occupancy Model III. Resurrecting Efficacy. *Journal of Theoretical Biology* 181:381-397.

Wellendorph P, Goodman MW, Burstein ES, Nash NR, Brann MR and Weiner DM (2002) Molecular cloning and pharmacology of functionally distinct isoforms of the human histamine H(3) receptor. *Neuropharmacology* 42:929-940.

Welsby PJ, Kellett E, Wilkinson G and Milligan G (2002) Enhanced detection of receptor constitutive activity in the presence of regulators of G protein signaling: applications to the detection and analysis of inverse agonists and low-efficacy partial agonists. *Mol Pharmacol* 61:1211-1221.

West RE, Jr., Moss J, Vaughan M, Liu T and Liu TY (1985) Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. *J Biol Chem* 260:14428-14430.

Wettschureck N and Offermanns S (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85:1159-1204.

Wiedemann P, Bonisch H, Oerters F and Bruss M (2002) Structure of the human histamine H_3 receptor gene (HRH3) and identification of naturally occurring variations. *J Neural Transm* 109:443-453.

Wieland K, Bongers G, Yamamoto Y, Hashimoto T, Yamatodani A, Menge WM, Timmerman H, Lovenberg TW and Leurs R (2001) Constitutive activity of histamine h(3) receptors stably expressed in SK-N-MC cells: display of agonism and inverse agonism by H(3) antagonists. *J Pharmacol Exp Ther* 299:908-914.

Wijtmans M, Leurs R and de Esch I (2007) Histamine H3 receptor ligands break ground in a remarkable plethora of therapeutic areas. *Expert Opin Investig Drugs* 16:967-985.

Wilker E and Yaffe MB (2004) 14-3-3 Proteins--a focus on cancer and human disease. *J Mol Cell Cardiol* 37:633-642.

Williams JG (1999) Serpentine receptors and STAT activation: more than one way to twin a STAT. *Trends Biochem Sci* 24:333-334.

Wise A, Gearing K and Rees S (2002) Target validation of G-protein coupled receptors. *Drug Discov Today* 7:235-246.

Witte DG, Yao BB, Miller TR, Carr TL, Cassar S, Sharma R, Faghih R, Surber BW, Esbenshade TA, Hancock AA and Krueger KM (2006) Detection of multiple H_3 receptor affinity states utilizing [^{3}H]A-349821, a novel, selective, non-imidazole histamine H_3 receptor inverse agonist radioligand. *Br J Pharmacol* 148:657-670.

Wong SK (2003) G protein selectivity is regulated by multiple intracellular regions of GPCRs. *Neurosignals* 12:1-12.

Wulff BS, Hastrup S and Rimvall K (2002) Characteristics of recombinantly expressed rat and human histamine H_3 receptors. *Eur J Pharmacol* 453:33-41.

Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, Wang QP, Tominaga M, Goto K, Shioda S and Sakurai T (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. *Biochem Biophys Res Commun* 290:1237-1245.

Yamashita M, Fukui H, Sugama K, Horio Y, Ito S, Mizuguchi H and Wada H (1991) Expression cloning of a cDNA encoding the bovine histamine H_1 receptor. *Proc Natl Acad Sci U S A* 88:11515-11519.

Yan Z, Hsieh-Wilson L, Feng J, Tomizawa K, Allen PB, Fienberg AA, Nairn AC and Greengard P (1999) Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. *Nat Neurosci* 2:13-17.

Yanai K, Ryu JH, Sakai N, Takahashi T, Iwata R, Ido T, Murakami K and Watanabe T (1994) Binding characteristics of a histamine H_3-receptor antagonist, [^{3}H]S-methylthioperamide: comparison with [^{3}H](R)alpha-methylhistamine binding to rat tissues. *Jpn J Pharmacol* 65:107-112.

Yang R, Hey JA, Aslanian R and Rizzo CA (2002) Coordination of histamine H3 receptor antagonists with human adrenal cytochrome P450 enzymes. *Pharmacology* 66:128-135.

Yao BB, Sharma R, Cassar S, Esbenshade TA and Hancock AA (2003) Cloning and

pharmacological characterization of the monkey histamine H_3 receptor. *Eur J Pharmacol* 482:49-60.

Zhu Y, Michalovich D, Wu H, Tan KB, Dytko GM, Mannan IJ, Boyce R, Alston J, Tierney LA, Li X, Herrity NC, Vawter L, Sarau HM, Ames RS, Davenport CM, Hieble JP, Wilson S, Bergsma DJ and Fitzgerald LR (2001) Cloning, expression, and pharmacological characterization of a novel human histamine receptor. *Mol Pharmacol* 59:434-441.

Curriculum Vitae

Gerold Bongers was born in Oss on November 19th 1976. He finished the athenaeum at the Commenius College in Uden in 1995 and in the same year, he started as a biopharmaceutical sciences student at the State University Leiden.

In 1996 he started as a chemistry student at the Vrije Universiteit Amsterdam, where he did his minor at the department of Biochemisty under supervision of Dr. Marco Siderius and studied cross-talk of two osmosensing MAP kinase pathways in the yeast *Saccharomyces cerivisiae*.
His major research was done in the lab of Dr. Rob Leurs in the department of Pharmacochemistry at the Vrije Universiteit Amsterdam under supervision of Kerstin Wieland. There, he studied the effects of sodium and the constitutive activity of the histamine H_3 receptor, as well as the species differences between the rat and human histamine H_3 receptor.
In 2000 he worked for seven months in the lab of Dr. Lennart Mucke in San Francisco, USA at the Gladstone Institute of Neurological Disease under supervision of Dr. Jacob Raber. There, he studied the protective effects of androgens against apolipoprotein E4-induced cognitive deficits and the role of H_3-receptor-mediated signaling in anxiety and cognition in wild-type and ApoE$^{-/-}$ mice.

After his graduation in 2002 he started his Ph.D. in the department of Pharmacochemistry at the Vrije Universiteit under supervision of Dr. R. Bakker and Dr. R. Leurs. There, he studied the signal transduction of the histamine H_3 receptor and the results can be found in this thesis. From 2006 to 2008 he worked as a post-Ph.D/pre-doc at the same department.

In 2008 he started his post-doc at the Mount Sinai Medical School, New York, USA on the *in vivo* characterization of the virally encoded chemokine receptor US28.

List of publications

Bongers G, Krueger KM, Miller TR, Baranowski JL, Estvander BR, Witte DG, Strakhova MI, van Meer P, Bakker RA, Cowart MD, Hancock AA, Esbenshade TA, Leurs R. A 80 amino acid deletion in the third intracellular loop of a naturally occurring human histamine H_3 isoform confers pharmacological differences and constitutive activity. J Pharmacol Exp Ther. 2007, 323(3), 888-98.

Bongers G, Sallmen T, Passani MB, Mariottini C, Wendelin D, Lozada A, Marle AV, Navis M, Blandina P, Bakker RA, Panula P, Leurs R. The Akt/GSK-3β axis as a new signaling pathway of the histamine H_3 receptor. J Neurochem. 2007, 103(1), 248-58.

Bongers G, Bakker RA, Leurs R. Molecular aspects of the histamine H_3 receptor. Biochem Pharmacol. 2007, 73(8), 1195-204.

Kitbunnadaj R, Hoffmann M, Fratantoni SA, **Bongers G**, Bakker RA, Wieland K, el Jilali A, De Esch IJ, Menge WM, Timmerman H, Leurs R. New high affinity H_3 receptor agonists without a basic side chain. Bioorg Med Chem. 2005, 13(23), 6309-23.

Bongers G, LeFevour A, Robertson J, Raber J. Role of H_3 receptor-mediated signaling in cognition. Inflamm Res. 2004, Suppl 1,S51-2.

Bongers G, Leurs R, Robertson J, Raber J. Role of H_3-receptor-mediated signaling in anxiety and cognition in wild-type and $Apoe^{-/-}$ mice. Neuropsychopharmacology. 2004, 29(3), 441-9.

Raber J, **Bongers G**, LeFevour A, Buttini M, Mucke L. Androgens protect against apolipoprotein E4-induced cognitive deficits. J Neurosci. 2002, 22(12), 5204-9.

Wieland K, **Bongers G**, Yamamoto Y, Hashimoto T, Yamatodani A, Menge WM, Timmerman H, Lovenberg TW, Leurs R. Constitutive activity of histamine H_3 receptors stably expressed in SK-N-MC cells: display of agonism and inverse agonism by H_3 antagonists. J Pharmacol Exp Ther. 2001, 299(3),908-14.

List of abbreviations

[^{35}]GTγS	guanosine-5′-O-(3-[^{35}S]thio)triphosphate
[Ca^{2+}]$_i$	intracellular calcium
2-APB	2-aminoethoxydiphenyl borate
A-304121	(R)-2-Amino-1-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-propan-1-one
A-317920	furan-2-carboxylic acid ((R)-2-{4-[3-(4-cyclopropanecarbonyl-phenoxy)-propyl]-piperazin-1-yl}-1-methyl-2-oxo-ethyl)-amide
A-320436	furan-2-carboxylic acid ((R)-2-{4-[3-(4'-cyano-biphenyl-4-yloxy)-propyl]-[1,4]diazepan-1-yl}-2-oxo-1-thiazol-4-ylmethyl-ethyl)-amide
A-331440	4'-[3-((R)-3-Dimethylamino-pyrrolidin-1-yl)-propoxy]-biphenyl-4-carbonitrile
A-349821	{4'-[3-((2R,5R)-2,5-Dimethyl-pyrrolidin-1-yl)-propoxy]-biphenyl-4-yl}-morpholin-4-yl-methanone
A-358239	4-{2-[2-((R)-2-Methyl-pyrrolidin-1-yl)-ethyl]-benzofuran-5-yl}-benzonitrile
A-431404	(4-Fluoro-phenyl)-{2-[2-((R)-2-methyl-pyrrolidin-1-yl)-ethyl]-benzofuran-5-yl}-methanone.
AC	adenylyl cyclase
ADHD	attention deficit hyperactivity disorder
AG1478	(N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine)
Akt inhibitor	(1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate)
ATP	adenosine triphosphate
BAPTA-AM	(1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
B_{max}	maximal specific binding of a ligand
BSA	Bovine serum albumin
cAMP	cyclic 3',5'-adenosine monophosphate
CHO	Chinese hamster ovary
CNS	central nervous system
COS-7	SV40 immortalised African green monkey kidney
CTC model	cubic ternary complex model
DMEM	Dulbecco's Modified Eagle Medium
EC_{50}	molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EMEM	Eagle's minimal essential medium
ER	endoplasmic reticulum
Erk1/2	extracellular signal-regulated kinase1/2
FUB-322	3-(1H-imidazol-4-yl)propyl-di(p-fluorophenyl)-methyl ether hydrochloride
G protein	guanine nucleotide-binding protein
GPCR	G-protein coupled receptor

GSK-3	Glycogen synthase kinase 3
GTPγS	guanosine-5′-O-(3-thio)triphosphate
H_3R	histamine H_3 receptor
hH_3R	human histamine H_3 receptor
IL3	third intracellular loop
IPP	Iodophenpropit
KAP	kinase-activated protein
K_d	equilibrium dissociation constant of a ligand determined directly in a binding assay using a labeled form of the ligand
K_i	equilibrium dissociation constant of a ligand determined in inhibition studies
MAP	mitogen-activated protein
MAPK	Mitogen-activated protein kinase
NαMH	N^{α}-methylhistamine
NBM	neurobasal medium
NHE	Na^+H^+ exchanger
pA_2	negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of the agonist needed to elicit the original submaximal response obtained in the absence of antagonist
PBS	phosphate buffer saline
PI3K	phosphoinositide-3 kinase
PKA	protein kinase A
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSF	mM phenylmethylsulfonyl fluoride
PTX	pertussis toxin
RIPA	radioimmunoprecipitation assay
R-SAT	receptor selection and amplification technology
TBS	Tris buffered saline
TM	transmembrane domain
tr-FRET	time-resolved Fluorescent Resonance Energy Transfer
T-TBS	Tris buffered saline containing tween
HBSS	Hanks Balanced Salt Solution