CHAPTER 07

DISCUSSION AND CONCLUSION

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This concluding chapter will summarize and discuss the results of this thesis. Although the focus of the project is by no doubt the histamine H_4 receptor (H_4R), in this final chapter I will link to results from other GPCR studies. Indeed, one cannot directly compare two different GPCRs, however one can extract information on ligand binding, activation mechanism and biased behaviour from studies throughout the GPCR family and eventually translate this to the H_4R. The research projects discussed below were initiated in order to reach three goals:

❶ validate H_4R ligand binding mode and identify activation requirements.
❷ identify biased H_4R ligands and unravel the structural needs for ligand bias.
❸ assay optimization to allow novel (biased) H_4R ligand discovery.

❶ VALIDATE H_4R LIGAND BINDING MODE AND IDENTIFY ACTIVATION REQUIREMENTS.

Ligand binding mode(s) for aminergic (rhodopsin family) GPCRs have been investigated extensively. The binding cavity that is formed by the 7TM helices can generally be divided in two subpockets. A minor pocket is located between TM1, 2, 3 and 7 and a major pocket is formed by TM3, 4, 5 and 6[150]. Side-directed mutagenesis (SDM) approaches have identified amino acid residues that play an important role in ligand binding. Four structural hallmarks of the aminergic receptor family are of major importance: I - the conserved ionic interaction anchor $D^{3.32}$ in $TM3^{318}$; II - the aromatic cluster in $TM6^{319\text{-}321}$; III - functional receptor selectivity via $TM5^{154}$ and IV - allosteric contacts with the minor pocket and extracellular loops[271].

BINDING MODES FOR H_4R LIGANDS

Amino acid residues $D^{3.32}$ and $E^{5.46}$ are the key anchor points for histamine binding to H_4R. $D^{3.32}$ interacts with the amine group of histamine, whereas $E^{5.46}$ forms an hydrogen-bond and ionic interaction with the imidazole nitrogen[153,322,153,323,271] (Figure 1-11, 2-1). The majority of published H_4R ligands interact with these two important residues and with the polar pocket between $S^{6.52}$ and $T^{6.55}$ [152,157].

Initially, these binding modes were deduced on the basis of SDM studies, but in the last decade computational approaches have proven to be a fruitful addition. At first, the rhodopsin crystal structure was used as template to generate a H_4R homology model, but in 2007 the structure of the more closely related bioaminergic β2-adrenergic receptor (ADRB2,) became available and replaced rhodopsin as H_4R template. Only recently the histamine H_1 receptor (H_1R) structure was elucidated, which now functions as nearest H_4R template. It has to be noticed, however, that this receptor is still quite different from H_4R, as illustrated by the relatively low amino acid

sequence similarity (e.g. there is higher sequence similarity between H_1R and M_2R/M_3R than between the binding sites of H_1R and H_3R/H_4R[271]). The H_1R-based homology model could best explain the 2-aminopyrimidine and indolecarboxamide binding modes, in which the aminopyridimes can accommodate larger substituents than the indolecarboxamides[179].

It has to be noted that 3D X-ray images from crystalized receptors are obtained under harsh experimental conditions (i.e. detergents), which require engineered mutant receptors or GPCRs fused to large stable proteins[324]. Moreover, the reported structures are snap shots of dynamic proteins and consequently still need to be verified and validated with experimental data. We therefore use a multidisciplinary approach that combines H_4R SDM studies, with molecular dynamics simulations and homology model docking studies. Using this approach we previously investigated the binding properties of different structural H_4R ligand classes[151,157]. Binding modes for clobenpropit analogues[151], aminopyrimidines and indolecarboxamides (e.g. JNJ 7777120)[157] have been published. It was shown that clobenpropit can bind to H_4R in two different ways, whereas an analogue that bears an additional cyclohexyl group at the isothiourea moiety can only bind in one specific mode[151]. Both the aminopyrimidines and indolecarboxamides have a single binding orientation, in which their basic methylpiperazine interacts with $D^{3.32}$ in TM3[157] (see also Chapter 1).

RATIONAL DESIGN OF VUF14480

Chapter 2 of this thesis nicely demonstrates that our current knowledge of the H_4R ligand-binding pocket has increased to such extent that we can rationally design a ligand with specific receptor interactions. Based on the previously elucidated binding mode of 2-aminopyrimidines[157] (see previous section), the close proximity between the 2-amino group of these ligands and the $C^{3.36}$ residue in TM3 was proposed (Figure 2-1). This hypothesis led to the design and synthesis of a 2-aminopyrimidine analogue (VUF14480) in which the 2-aminogroup was replaced by an electrophilic Michael acceptor (ethylene group) to allow an interaction with the reactive thiol group (nucleophile) of $C^{3.36}$ if they are indeed in close proximity. We showed that VUF14480 forms a covalent interaction with thiols like glutathione or N-acetyl cysteine, whereas a methyl analogue did not react in a covalent manner (Figure 2-2). Using a newly designed preincubation with VUF14480 and a pharmacological wash-out binding study we identified a concentration-dependent loss in histamine binding sites (Figure 2-4). Mutation of $C^{3.36}$ in a serine residue showed that this loss in histamine binding sites was dependent on the interaction with the thiol group (Figure 2-6). An irreversible interaction between VUF14480 and H_4R was therefore most likely (Figure 2-7).

This observation demonstrates the validity of our previously proposed H_4R ligand binding models. Importantly, these models can in future research complement our recent fragment screen data and correlate structural features to H_4R activity (Chapter 4 and 5). Moreover, the irreversible H_4R ligand VUF14480 can be a very interesting tool in H_4R crystallization efforts.

VUF14480 IS A PARTIAL AGONIST, WHAT DOES THIS TEACH US ABOUT RECEPTOR ACTIVATION?

We investigated the functional efficacy of VUF14480, which resulted in the identification of partial agonism. Notable, the original 2-aminopyrimide was found to be an H_4R antagonist (Figure 2-3), illustrating that we have identified an interesting ligand activity cliff, but possibly also a H_4R activation switch in the vicinity of H_4R-$C^{3.36}$. The subtle efficacy change from an aminopyrimidine antagonist into an irreversible partial agonist (VUF14480) could be caused by its interaction with $C^{3.36}$. This hypothesis was further supported by the $C^{3.36}S$ mutation resulting in a lower efficacy for VUF14480 (Figure 2-5). Is $C^{3.36}$ a key interaction for H_4R activation or are more residues important? To answer this question we first broaden our view to other GPCRs. How does receptor activation work for the rhodopsin family members?

Both active and inactive X-ray structures of the ADRB2 receptor are nowadays available[62,63,74,185]. Comparison of these structures highlights subtle differences that could be the consequence of agonist binding/stabilization. The active structure shows an outward movement of TM5 and 6 and an inward movement of TM3 and TM7 at the intracellular site as compared to the inactive structure[71]. Although this indicates what happens to the receptor conformation, this does not specify the exact amino acids that cause these helix movements. A recent study summarized activation data for several GPCRs and identified amino acid positions that correlated with agonism or antagonism, as well as specific ligand properties[318,325]. Amino acid residues responsible for antagonist binding are found in the extracellular regions of TM5 / TM6 or are located at the interface of TM5 and TM6. Regions that are important for agonist binding seem to correlate to residues that position their side chain between TM1, TM2 and TM3[319-321,325]. Residues in EL2 (close to the conserved cysteine that is involved in the disulphide bond with $C^{3.25}$) all play an important role in agonist binding[325]. The serotonin 5-HT_4 receptor is believed to be stabilized in an inactive state due to intramolecular interactions between $D^{3.32}$, $W^{6.48}$ and $F^{6.51}$[326]. In ADRB2, $W^{6.48}$ is proposed as molecular switch that triggers receptor activation by modification of the rotameric states of aromatic residues. A subsequent change in $P^{6.50}$ bends TM6 and disrupts the ionic lock between $R^{3.50}$, $D^{3.49}$ and $E^{6.30}$[327,328]. Originally, such conserved motifs (i.e. ionic locks, rotamer toggle switches) were thought to be important in GPCR activation. However, these SDM-based theories do not always corroborate with the now available active and inactive GPCR structures[329].

A consensus thus far can be found in the statement that intramolecular interactions keep the receptor in its inactive conformation[57,330-332]. Agonist binding is believed to disrupt these constraints, which results in conformational changes that finally lead to an active receptor state[325]. Hence, a single residue ($C^{3.36}$) as activation switch seems not likely, but the conformational change induced/forced by the covalent interaction with VUF14480 could disrupt basal intramolecular interactions. The importance of position 3.36 in receptor activation was previously illustrated for H_1R[195].

H_1R activation

In 2005 our group proposed the first steps in the activation mechanism for the histamine H_1R[195]. Binding of histamine to the H_1R changes the conformation of $S^{3.36}$ side chain from inactive state (g- or t), which points in the direction of TM6, to an active g+ state that facilitates hydrogen bonding with the protonated amine of histamine. Meanwhile, this protonated amine can also form a hydrogen bond with $D^{3.32}$, which forces a change in the orientation of $S^{3.36}$ toward TM7. In the active H_1R state $S^{3.36}$ forms a hydrogen bond with $N^{7.45}$, which is suggested to function as toggle switch[195]. Interestingly, a water molecule is needed to keep the intramolecular hydrogen-bond network in an inactive H_1R intact[195]. The function of water molecules in GPCR binding pockets has for long been neglected, but recently regained attention.

Role of water molecules in GPCR structure and activation

Water molecules play a crucial role in the conformation and stabilization of proteins. It is known that ordered water molecules in GPCR are conserved and that they are presumably located in the vicinity of polar or charged amino acid networks[333]. The X-ray structure of rhodopsin showed a crucial role for water molecules in the hydrogen-bond network between TM6 and 7[334,335]. Water molecules are suggested to fill the gap between residues that are unable to interact directly[335,336]. In addition, several other GPCR X-ray structures showed water molecules in the hydrophobic core interacting with conserved residues[336], conserved motifs (e.g. NPxxY)[337] and hydrogen-bond networks[64,338]. Moreover, the in and outflow of water molecules in the cavities formed by the 7TM helices could be an explanation for the equilibrium between inactive and active states and the level of constitutive receptor activity.

ACTIVATION MECHANISM OF H_4R

Previously, our group investigated the molecular basis for agonist binding to the H_4R. Interestingly, it was indicated that agonists create a perfect orientation of hydrogen bonds and stabilize the rotamer state of $W^{6.48}$. In addition, an important interaction between $E^{5.46}$ and $W^{6.48}$ was highlighted[152]. In earlier studies a role for $N^{4.57}$ and $S^{6.52}$ in H_4R activation was demonstrated[153], which further illustrates an apparently crucial role for polar interactions in H_4R structure (see also Chapter 1). Although these interactions are not directly linked to $C^{3.36}$, a covalent interaction with this residue could well disrupt the hydrogen bond network and thereby initiate H_4R activation. This is moreover illustrated by our other studies involving H_4R agonists.

In chapter 4 we hypothesized that the non-biased (balanced) agonist indolecarboxamide **75** (a JNJ 7777120 analogue with a nitro group at the aromatic indole moiety) uses polar interactions in the vicinity of TM5 that are crucial for G protein agonism. In other studies that involved H_4R ligands (e.g. clobenpropit analogues, quinolone-based tricyclic compounds) we observed changes in functionality upon mutation of the TM5 region (unpublished results). These observations let to the hypothesis that H_4R agonists interact with a polar network in the vicinity of $E^{5.46}$ and polar TM5 residues $T^{5.42}$ and $S^{5.43}$. However, mutation of the polar residues into non-polar variants was

not sufficient to completely loose efficacy. This is indeed further demonstrated by the fact that indolecarboxamide **75** is still able to activate the receptor even when both polar groups ($S^{5.43}$ and $T^{5.42}$) are removed (unpublished results). Because the polar residues in the H_4R TM domains are not all in close enough proximity to directly interact, water molecules could function as hydrogen-bond linkers. This area has not been investigated for the H_4R, but is worthwhile to further explore.

❷ IDENTIFY BIASED H_4R LIGANDS AND UNRAVEL THE STRUCTURAL NEEDS FOR LIGAND BIAS.

EXTRA COMPLICATIONS DUE TO BIASED RECEPTOR ACTIVATION

While the exact mechanism for GPCR activation still remains to be elucidated, GPCR research has become even more complicated following the discovery of biased receptor signalling. The GPCR is in fact not a rigid lock that binds an unbendable key, but rather a dynamic entity that can easily adjust its conformation and allow interactions with flexible ligands[339]. Consequently, GPCRs can adopt not just one active or inactive state, but multiple active and inactive conformations[258] that subsequently lead to differential downstream signalling[50]. GPCR-mediated signalling is thus not linear but rather a pluridimensional network in which some ligands activate only a subset of the known signalling pathways and thereby display biased agonism. Several ligand-GPCRs combinations have been shown to display biased signalling[340] (see also therapeutic potential section).

Although in literature the majority of papers report biased signalling between G protein- and arrestin-dependent signalling pathways, also other types of biased behaviour are known. Bias for different G protein subtypes[341] or β-arrestin isoforms, and even different internalisation mechanisms exist, but will not be discussed in this thesis.

BIASED SIGNALING OF H_4R

Histamine-stimulation of the H_4R results in $G\alpha_i$ protein activation and $G\alpha_i$-independent recruitment of β-arrestin2 proteins[140]. Interestingly, JNJ 7777120 was identified as partial agonist in the β-arrestin recruitment assay, although this ligand was previously considered as neutral H_4R antagonists and used as reference compound in several studies. Further investigation of downstream signalling showed that H_4R-$G\alpha_i$ protein-mediated signalling in response to histamine resulted in a quick ERK phosphorylation (max at 2 min), whereas stimulation with the β-arrestin-biased JNJ 7777120 resulted in a later onset (20 min) of ERK phosphorylation[140].

In chapter 3 we set ourselves the challenge to re-analyse known H_4R ligands for their ability to signal in a biased manner. More explicitly, we compared efficacies of these ligands in two different readouts, the $G\alpha_i$ protein-mediated CRE luciferase assay and the $G\alpha_i$-independent

β-arrestin2 recruitment assay from DiscoveRx. A broad variety of efficacies was observed and subsequently we could identify ligands that were inhibitors in one pathway, but activated downstream signalling in the other pathway (full biased ligands). In addition, also ligands that showed agonism or antagonism in both pathways were found. Interestingly, no clear correlation was observed between biased behaviour and ligand class/structure. A more detailed investigation into the β-arrestin biased indolecarboxamides was therefore initiated that would allow the identification of subtle structural changes leading to altered functional efficacy (Chapter 4). Moreover, our latest effort has been the screening of 1009 fragment-like molecules for their binding and functional effects (i.e. G protein signalling and β-arrestin recruitment) on H_1R-H_4R. We have identified additional G protein biased or β-arrestin biased H_4R fragments that will form the basis for future investigations into the structural requirements for biased H_4R signalling (Chapter 5).

BIASED BINDING MODES - DIFFERENT DISRUPTION OF INTRAMOLECULAR CONSTRAINTS?

We analysed 48 indolecarboxamides (i.e. JNJ 7777120 analogues), with subtle structural changes, in β-arrestin recruitment and G protein signalling assays. Subsequent computational analysis identified ligand regions and receptor domains that are possibly involved in β-arrestin-biased H_4R signalling (Figure 4-6). Interestingly, one of the compounds (indolecarboxamide **75**, see activation mechanism of H_4R section) appeared to be a balanced (unbiased) H_4R agonist. The nitro group at the aromatic indole moiety of **75** nicely demonstrates the importance of a hydrogen-bonding network in receptor activation (see previous sections), but also showed that this polar group is apparently not needed for β-arrestin recruitment. Interestingly, recent biophysical studies on ADRB2 showed that β-arrestin-biased agonists have limited effects on GPCR structure[226,342,343], which is in line with our observations that β-arrestin biased ligands are less dependent on polar interactions and probably induce less conformational changes in the H_4R.
Trying to solve the mechanism for biased signalling is very challenging, although some research groups have made progress. Intramolecular BRET- or FRET-based biosensors allow the detection of minor structural changes in either intracellular regions of the GPCR[342] or interacting partners, such as β-arrestin2[344]. Different ligands (biased vs balanced) appear to stabilize not only distinct GPCR conformations, but also lead to functionally specific conformations in β-arrestin proteins[345]. Upon binding of full agonists, the ADRB2 conformation changes at C^{265} in TM6 and C^{327} in TM7. Partial agonists have smaller effects on TM6 but still move TM7 to an equal extent as full agonists. Neutral antagonist do not show major movements. Interestingly, β-arrestin-biased ligands seem to specifically change TM7 and not TM6. Furthermore, TM6 and TM7 can change their conformation independently upon binding of diverse ligands, which could be an underlying mechanism for biased agonism[261]. Ligands with different chemical structures have also been shown to induce different conformations in purified ADRB2 and influence the ionic lock and the rotamer toggle switch. However, this did not necessarily lead to different signalling

outcomes[57,346], again illustrating that only subtle structural changes induce biased signalling. Recently, it was postulated that the minor pocket between TM helices 1, 2, 3 and 7[57,347], which includes a conserved proline kink in TM2, plays an important role in determining the qualitative nature of ligand bias[347]. Another study focused on the identification of specific amino acids in GPCRs that are responsible for different signalling outcomes. To this end, several different ligand-receptor pairs were tested and data was correlated to the known G protein signalling pathway preference[325]. Specific amino acid positions could be extracted for $G\alpha_s$, $G\alpha_i$ and $G\alpha_q$ signalling. These residues are not only positioned in the TM regions, but extend to extracellular loop 2 and helix 8[325]. Only very recently crystal structures of ADRB1 bound to the biased agonists bucindolol and carvedilol became available[348]. Comparison of ADRB1 structures with full agonists, partial agonists, inverse agonists or biased agonists identified specific interactions with EL2 and TM7 that are presumably involved in biased receptor activation of ADRB1[349].

IS IT THE EXTRACELLULAR SIGNAL (LIGAND) OR INTRACELLULAR SIGNAL (DOWNSTREAM INTERACTORS) THAT DETERMINES BIASED SIGNALLING?

It has been proposed that biased signalling is not only caused by a difference in ligand binding, but is rather the consequence of different expression levels in downstream signalling components (e.g. G proteins, β-arrestin, cAMP and kinases)[50,350]. The influence of these factors on biased signalling will be discussed in this paragraph. GPCRs are widely distributed throughout our tissues, however their predominant localization is entangled within the dynamic cellular membrane. The membrane bilayer composition has great influence on the structure and function of its embedded proteins[351,352]. It is therefore surprising that so far not much attention is given to the function of this lipid environment on GPCR functioning. Only recently, the role of cholesterol on GPCR structure and function has been studied[353]. Some studies even speculate on specific cholesterol binding sites in GPCRs, although this remains debatable[354-356]. A cholesterol binding motif (tyrosine in TM2 and tryptophan, leucine as well as a charged residue at the intracellular side of TM4) is apparently present in the majority of rhodopsin family GPCRs[355]. Also H_4R seems to have this cholesterol binding motif, possibly formed by $Y^{2.41}$ in TM2 and $K^{4.41}$, $W^{4.50}$ and $L^{4.40}$ in TM4, which would indicate a possible role for this membrane component on H_4R functioning.

It is hypothesized that cholesterol locates the GPCRs to dedicated membrane domains (also known as lipid rafts or caveolae) in which colocalization with protein members of the signalosome occurs[353,357-361]. The existence of microdomains is still highly debatable, but there are studies that hint at their occurrence and suggest that they contribute to biased signalling[362]. The organized localization of G protein subtypes was suggested to be an important factor that determines the specific activation of downstream signaling by the oxytocin (OT) receptor[357]. The ability of OT receptors to signal via $G\alpha_{q/11}$ or $G\alpha_i$ proteins was dependent on the localization of the GPCR in the cholesterol rich microdomains in the membrane[357]. Also the Vasopressin receptor V1b signaling has been shown to be dependent on membrane compartmentalization,

where localization into cholesterol-rich domains ensures coupling to $G\alpha_{q/11}$[350]. In contrast, V1b localization in non-cholesterol rich regions ensures interaction with $G\alpha_{s}$[353].
In addition to cholesterol, also palmytoylation of cysteine residues in the C-terminal helix 8 could play a role in the targeting of GPCRs to microdomains[363]. Interestingly, also the H_4R has a cysteine residue in its presumed helix 8 (snake plot in Figure 1-8) and is thus potentially subjected to microdomain targeting.

PHOSPHORYLATION STATUS DETERMINES BIASED SIGNALLING?

Upon receptor activation, heterologous desensitization occurs after GPCR phosphorylation by protein kinase C (PKC) or protein kinase A (PKA)[364,365]. Consequently, this desensitization can also be triggered by kinases activation through non-GPCR mediated pathways[366]. Homologous desensitization is regulated by GPCR kinases (GRKs) [367] or casein kinase 1a[368] that are able to phosphorylate specific residues at the intracellular side of the GPCR[369]. GRKs are known to play a key role in regulating GPCR desensitization, internalization and as starting point for G protein-independent signalling[24]. Phosphorylated GPCRs can interact with β-arrestin proteins in a transient or sustained manner, resulting in rapid recycling or degradation of the GPCR, respectively. However, also correlations with activation of specific signaling pathways were reported. A correlation was found between agonist-induced phosphorylation and μ opioid receptor (MOR) biased signalling. Low levels of MOR phosphorylation led to activation of the PKC pathway. In contrast, high levels of MOR phosphorylation resulted in activation of the β-arrestin pathway[370]. Interestingly, β-arrestin-biased synthetic ligands have been developed for the AT1aR, ADRB2 and PTH receptor[51,371-373] (see also therapeutic potential section). These ligands cause GPCR phosphorylation and β-arrestin-mediated signaling without activation of G proteins[4,374].
Considering the high GRK specificity of GPCRs, it could be that β-arrestin biased signaling is regulated by specific GRK-GPCR interactions[375]. Evidence for GRK-directed signaling has come from studies with the AT1aR, V2R, ADRB2, and FSH-R, in which GRK5 and GRK6 were involved in β-arrestin2-dependent phosphorylation of MAPK pathway components[376-379]. In contrast, upon V2R[377] and H_1R[380] activation, GRK2 negatively regulates second messenger generation, whereas no role for GRK5 or GRK6 was found. ADRB2 GKR2 phosphorylation sites are involved in receptor endocytosis, whereas GRK6 is important for β-arrestin2 mediated ERK activation. Activation of chemokine receptor CCR7 by CCL19 leads to GRK3- and GRK6-mediated phosphorylation and subsequent β-arrestin2 recruitment. In contrast, CCL21 only induced CCR7 phosphorylation by GRK6. Consequently, both ligands initiated signaling to ERK kinases, but only CCL19-induced signaling leads to receptor endocytosis[375]. GRKs seem to be important determinants in biased GPCR signaling by providing an intracellular phosphorylation bar code[22,367,376,378].
The C-terminal region of H_4R also contains threonine and serine residues that might be phosphorylated by specific GRKs. Differences in phosphorylation patterns upon biased ligand stimulation could be the initial step in biased H_4R signaling. This hypothesis is currently being investigated.

DO BIASED LIGANDS ACTIVATE A NEW SIGNALLING PATHWAY OR A SUBSELECTION OF THE PATHWAYS ACTIVATED BY THE ENDOGENOUS LIGAND?

The study into the mechanism of β-arrestin-biased SII at the AT1A receptor clearly demonstrates our limited knowledge of biased signalling. Using BRET probes that directly measure G protein activity it was discovered that SII-induced β-arrestin2 recruitment is partially $G\alpha_q$ dependent in recombinant HEK293T cells. Likewise, ERK phosphorylation downstream of β-arrestin appeared to be dependent on $G\alpha_i$ and $G\alpha_q$ in primary MEF cells. Hence, SII activates the AT1R receptor via a mechanism that is not used by the endogenous agonists Angiotensin II. This study is the first indication that biased agonists not necessarily activate a selection of endogenous signaling pathways, but can induce a completely new signaling pathway[381]. In contrast to angiotensin II, SII promotes receptor phosphorylation selectively by GRK6[381] apparently as a consequence of G protein-mediated signaling. However, previous reports suggested that GRKs are also able to phosphorylate GPCRs in absence of G protein coupling. This is further demonstrated by the biased CXCR7 receptor that does not couple to G proteins, but is able to recruit β-arrestin proteins to the receptor[382,383]. In addition, biophysical measurements of GPCR conformations indicate that ligands stabilize different active receptor states. Biased ligands could favour those conformations that normally are a minor proportion of the natural agonist conformations. Since the GPCR is inevitable subjected to the same structural constraints, it is not likely that novel receptor conformations exist that couple the receptor to unknown signaling routes. Also, extensive global proteomic approaches have not been able to identify specific response exclusively activated by biased ligands[384]. Separate biased signal transduction pathways are thus still debatable, especially since most probably not all downstream activation pathways of endogenous agonists are characterized.

Thus far, only H_4R signaling via $G\alpha_i$ and β-arrestin2 is demonstrated. β-arrestin signaling was long unknown, and only identified after the development of assays that could measure the recruitment of these proteins. β-arrestin recruitment is also observed after stimulation with the endogenous ligand histamine and therefore does not represent a separate pathway, but rather a selection of the endogenous ligand. It remains to be determined if H_4R has additional interaction partners and if those interactions are modulated by histamine. Only then we can conclude whether H_4R signal bias is a subset or a new signaling entity.

IS THERE MORE THAN β-ARRESTIN?

We have shown that H_4R can signal via two distinct pathways, namely pertussis toxin (PTx) sensitive heterotrimeric $G\alpha_i$ proteins and PTx-insensitive β-arrestin2[140,175]. In addition, we studied the interaction of H_4R with β-arrestin1 in a BRET assay. H_4R was fused to the optimized bioluminescent enzyme Renilla luciferase8 and the β-arrestin1 to the Yellow fluorescent protein. The Rluc8-YFP pair shows considerable overlap in emission and excitation spectra, which ensures the capability of energy transfer when these proteins are in close enough (1 nm-10 nm) proximity

and in a correct orientation. Close proximity between H_4R-Rluc8 and β-arrestin1-YFP proteins results in close proximity between Rluc8 and YFP. Subsequent energy transfer occurs, which than results in a BRET signal. We showed that β-arrestin1 is recruited to the H_4R upon receptor stimulation with a variety of ligands. Interestingly, the potency of all tested ligands to recruit β-arrestin1 is comparable to the potency to recruit β-arrestin2 (unpublished results).
Other GPCRs interact with several proteins that are involved in trafficking, localization and functioning (fine-tuning) of GPCRs[24,385]. Due to their ability to interact with such a variety of proteins, some previously described theories suggest that GPCRs and their effectors are localized in specialised microdomains. Many proteins function as scaffolding proteins that help to bring interacting proteins in close proximity into so called signalosomes[385]. Evidence for the existence of microdomains came from the identification of caveolin 3 as interaction partner of ADRB2 in myocyte membranes[386]. Other interacting proteins are: Regulators of G protein signaling (RGS), small GTPases, GPCR-associated sorting proteins (GASPs), receptor activity-modifying proteins (RAMPs), sphinophilin and Homer[24].
Intracellular interference with GPCR signaling is another potential way to modulate a receptor and could be an interesting option for therapeutic intervention. Specific blockage in the recruitment of intracellular interaction partners may be a selective way to modulate (bias) receptor function. However, one large drawback is the difficulty to pass the cell membrane[24]. Pepducins are potentially very interesting as intracellular protein modulators, since they are able to cross the membrane and bind to the intracellular surface of the GPCRs[387,388].
A recent publication reported an *in silico* method to unravel H_4R interaction partners in the brain[389]. Nonetheless, no experimental data has thus far accompanied this study and the potential interactions remain to be confirmed. We have made a start to investigate new interaction partners for H_4R by making use of a yeast-based assay system. Via this yeast screen, a number of potential interaction partners were found that are currently under investigation (unpublished results, Nijmeijer, Vischer, and Siderius).

HIGHER ORDER STRUCTURES?

GPCRs are able to form higher order oligomers consisting of two or more receptors that influence GPCR trafficking, functioning and signalling[390-392]. Could it be that biased signalling is the consequence of different higher order GPCR complexes?
We studied the interaction of H_4R with potential GPCR partners with a BRET assay. With this BRET method, H_4R has been shown to form both homodimers and heterodimers with H_1R and the EBV-encoded GPCR BILF1[103,122]. Moreover, we have identified CMV-encoded GPCR US28 and chemokine receptors CCR1, CXCR2, CXCR3, CXCR4 and CX3CR1 as dimerization partners for the H_4R (unpublished results).
It is still debatable if receptor monomers or receptor dimers are the functional signalling entity. To test whether H_4R dimers can recruit β-arrestin proteins, we used a combination of bimolecular protein complementation and BRET. The H_4Rs were fused to either the N- or C-terminal

part of Rluc8, whereas the β-arrestin protein is fused to an mVenus protein. Only after close proximity between the H_4R protomers a reconstitution of the Rluc8 can occur. This functional bioluminescent protein can subsequently transfer energy to mVenus if β-arrestin is recruited to the H_4R. From our data it can be observed that β-arrestin is recruited to the H_4R dimeric complex upon histamine activation. This indicates that a dimeric H_4R complex has a functionally active role in H_4R signalling (unpublished results).
Interestingly, further investigation into the H_4R-CXCR4 heterodimer in a YFP-complementation assay showed that stimulation with the endogenous agonists, histamine or chemokine CXCL12, resulted in internalization of the H_4R-CXCR4 heterodimer (unpublished results, Nijmeijer and Vischer). This observation adds another layer of complexity to GPCR signalling. Ligand cross talk, which depends on the co-expressed proteins, leads to changes in signal transduction that potentially could be interpreted as biased signalling.

❸ ASSAY OPTIMIZATION TO ALLOW NOVEL (BIASED) H_4R LIGAND DISCOVERY.

GPCR ASSAYS AND THEIR INFLUENCE ON RECEPTOR SIGNALLING AND CLASSIFICATION OF BIASED LIGANDS

Prior to our collaboration with Rosethorne and Charlton[175,266], we independently investigated the ability of H_4R to couple to the β-arrestin2 pathway. We used two different experimental techniques to measure β-arrestin recruitment to the receptor (i.e. BRET and Tango assay (Invitrogen)). We observed β-arrestin recruitment upon H_4R activation by histamine, but remarkably cannot measure β-arrestin recruitment upon JNJ 7777120 stimulation in both our assay formats (unpublished results). We are thus far unable to explain these discrepancies in assay results. Comparable to Rosethorne and Charlton[140], no constitutive activity or inverse agonism could be detected in the BRET-based β-arrestin recruitment assays. This could indicate that the H_4R does not recruit β-arrestin in a constitutive manner, which could be an interesting phenomenon. Previously, a similar observation has been reported for the H_1R, where the constitutive activity of the receptor initiates a different signal transduction route ($G\beta\gamma$) than the agonist-induced pathway ($G\alpha_{q/11}$)[393]. The lack of constitutive activity in the PathHunter assay could be caused by the fact that reconstitution of the β-galactosidase enzyme is not reversible, although this explanation is not valid for the FRET- or BRET-based assays. Importantly, assay systems that vary in their detection of constitutive activity may also lead to false biased identifications due to protean agonism[394]. A ligand that shows agonism in low constitutive activity systems (such as β-arrestin recruitment) may be identified as inverse agonist in systems with high constitutive activity[395]. An important consideration that we should take into account is the artificial setting in which β-arrestin recruitment is measured. These assays require engineered fusion proteins that can have major impact on protein conformation, activity and protein-protein interactions.

In contrast, reporter gene assays are very downstream in the signal transduction cascade, which makes them prone to amplification and subsequent overestimation of ligand efficacy. ^{35}S-GTPγS binding studies could serve as alternative $G\alpha_i$ protein readout, but depend on cell membrane suspensions instead of whole cells, which prevents GPCRs to interact with their intracellular components. This can potentially change the conformation, activation state and signalling of the GPCR[396] to such extend that no valid parameters (e.g. bias qualification) can be determined. A method to circumvent such problems is the use of GPCR-fusion proteins in which the GPCR of interest is fused to its intracellular signalling partner (i.e. G protein or β-arrestin). A research topic that is currently under investigation for the H_4R (unpublished results, Nijmeijer and Vischer). An elegant alternative for the previously described assays could be label free impedance or dynamic mass redistribution measurements, in which (biased) GPCR signalling can be measured in native cells. These methods were previously used to measure distinct signalling profiles of ADRB2 and CXCR3 ligands[397,398].

TRUE BIASED LIGANDS OR "BIASED" BIASED LIGANDS

Accurate definition of ligand bias is challenging, especially when different experimental systems are used. Differences in receptor reserve and amplification can lead to indistinguishable results between partial and full agonists in downstream assays. In contrast, upstream assays with a 1:1 stoichiometry result in a lower response for the partial agonists. Consequently, these differences can lead to incorrect conclusions concerning the biased behaviour of ligands. Black and white (i.e. agonist in pathway A, antagonist in pathway B) examples are easy to qualify as full biased compounds, however the greater challenge lies in compounds that show efficacy in both assays. How does one determine which downstream pathway is favoured by such compounds? Several different mathematical methods have been proposed and discussed these past years, which all have their different assumptions and limitations[44,399-402].

We have chosen for an analysis that is based on the operational model of agonism as originally described by Black and Leff[209] (Chapter 3). This model was designed to determine relative efficacies of agonists based on their concentration response curves. The operational model is especially useful when comparing two different assay systems. This method correlates the equilibrium dissociation constant (K_d or K_A) of a ligand to its coupling efficiency (τ). Tau includes both ligand efficacy and conditional efficacy as a consequence of the receptor density or coupling efficiency in the experimental system. Subsequent correlation of ligand τ values with τ values from a reference agonist makes it possible to compare relative τ values obtained from different assays[210,261]. The equilibrium dissociation constants of the respective ligands were determined in radioligand binding experiments. It is however important to realize that the absence or presence of specific buffer components (e.g. metal ions) can alter these values significantly (i.e different conditions between binding and functional assays). Consequently this can affect the outcome of the operational model analysis[403].

Recently, Kenakin and Christopoulos hypothesized that ligands have different affinities (i.e.

conditional affinity) for the receptor-interactor complex depending on the activated downstream signalling complex/pathway. They reported an adjusted operational model in which the arguable equilibrium dissociation constant (K_i) is not included in the equation, but where the conditional affinity is rather determined via the operational model function itself[402].

We have also tested this new analysis method for our H_4R biased ligand data set, but experienced some difficulties with the operational model fit. A possible explanation for this could be that the H_4R hardly shows any signal amplification (i.e. EC_{50} is not << K_i). For β-arrestin recruitment this observation has been reported previously, but for reporter gene assays this is very unusual. Since the operational model depends on this signal amplification, the question remains if we are allowed to fit our H_4R data to this model. Moreover, others have highlighted this drawback already for arrestin data that does not include an amplification step, but follows a 1:1 stochiometry[270] (see assay paragraph).

A relatively simple alternative method to compare ligand parameters is the use of intrinsic relative activity (iRA/RAi) values[404,405]. Here potency values (EC_{50}) are divided by the efficacy values (E_{max}) and correlated to a reference compound. In this method, the ligand concentrations that result in equiactive responses are correlated to the signalling response at equimolar concentration.

A matrix in which the biased factors (obtained from the operational model analysis) for all different readouts are summarized is useful if multiple downstream readouts are investigated. This matrix allows for a simple comparison of different ligands with their activity profile[22].

Investigations into the best method to analyse biased ligand data at the H_4R are currently ongoing and hopefully we can use our datasets to actively participate in this current discussion.

TRANSLATION FROM IN VITRO TO IN VIVO

Is the effect that we see *in vitro* (often in an isolated setting with recombinant proteins) a process that can be translated to an *in vivo* setting? It is still not clear what property determines the effect of a drug. Is it affinity, efficacy, a combination of both? Or is it all determined by kinetic parameters and is residence time the key factor?

In vivo, another level of complexity should also be taken into account: ligand metabolism. In chapter 6 we highlight the fact that ligands are subject to metabolic changes once entered in the human body. Instead of the effect of one stable ligand, we look at an overall effect of the parent compound and several of its metabolites. Importantly, the desired pharmacological effect of the parent compound can be completely abolished by metabolites with opposite functions. We therefore developed an in line fractionation and screening approach for metabolite mixtures to assess metabolite formation and their pharmacology in an early stage of drug discovery.

PHYSIOLOGICAL RELEVANCE AND THERAPEUTIC POTENTIAL?

Conventional drugs that interact with GPCRs either inhibit or mimic the action of the endogenous agonist. Consequently, all downstream pathways are affected which often also includes signalling routes that are not related to the treated disorder. As we now know, biased ligands do not affect these pathways to a similar extent, but preferentially modulate a subset of the responses. This implies that one can potentially design biased ligands that specifically target the disease-related signalling route, without interfering with non-disease related pathways. Such biased ligands are very promising therapeutics, although there are still some hurdles to take.

The selective targeting of the β-arrestin pathways has been proven to be beneficial for the ADRB1, AT1R and PTH receptors. For ADRB1, the β-arrestin activation is correlated to crosstalk with the EGFR resulting in a cardio protective outcome. The angiotensin ligand that distinctly activates the β-arrestin recruitment to the AT1R increases cardiac performance, without inhibiting unwanted metabolic side effects[54,206]. The β-arrestin biased PTH1 receptor agonist PTH7-24 has recently been investigated for its *in vivo* effect at the genomic level in mice[406,407]. The unbiased agonist affected genes that are associated with PTH action in regulation of skeletal morphogenesis, osteoblast differentiation and bone turnover. In contrast, the β-arrestin biased agonist inhibits this unwanted G protein signaling and signals to pathways that play a role in apoptosis and cell survival[406,407].

H_4R function has been tested in (native) cell types or animal models in which the final/overall ligand effect is measured. It is however not known which signalling pathways play a role in these physiological effects. The observed pharmacology is often an average effect that is caused by modulation of several different pathways. Interestingly, the discovery of $G\alpha_i$ protein independent β-arrestin2 recruitment to H_4R could indicate alternative signal transduction routes than previously thought. It remains to be investigated whether there are other H_4R signal transduction routes. Therefore, we are currently exploring possible interaction partners and potential signalling routes downstream of the H_4R (see arrestin section)

G protein biased ligands are suggested to be preferred as ligands that do not induce β-arrestin-mediated receptor desensitization and downregulation upon repeated dosing. Besides G protein-biased ADRB2 agonists for the treatment of asthma, G protein biased ligands at the μ opioid receptor (MOR) are also tested for their advantage compared to balanced ligands in producing analgesia without side effects (e.g. vomiting, constipation, tolerance and dependence)[408]. There is evidence that a G-protein-biased ligand such as herkinorin is an effective analgesic, but does not lead to significant tolerance and dependence[409,410,403,411].

The company Trevena specifically focuses on biased ligands as therapeutics. A β-arrestin biased AT1R agonist is currently in clinical phase II for the treatment of acute heart failure, whereas a G protein-biased MOR agonist, is in phase I for the treatment of post-operative pain. Other opioid receptor ligands are currently still in the pipeline.

In summary, there is huge potential for biased ligands as illustrated by the different examples above. What will be their potential use in H_4R-related diseases? It is often mentioned that inhibiting this receptor can be beneficial for immune disorders or asthma and pruritus. An interesting

observation that could be important for H_4R related signalling is the fact that anti-inflammatory effects on macrophages seem to be caused by β-arrestin-mediated signalling. This could potentially hint at a beneficial effect for β-arrestin biased H_4R ligands. However, another study reports that activation of the β-arrestin2 pathway in allergic airway inflammation is pro-inflammatory[270].
Animal data, of which it remains debatable if these are a correct representation of human (patho)physiology, suggest indeed that blocking H_4R relieves itch or inflammation. However, also conflicting data is reported. Moreover, how should we translate previous *in vivo* data of H_4R antagonists that now appear to be a β-arrestin biased agonist (i.e. JNJ 7777120). Are we looking at H_4R inhibition or are we actually looking at a biased effect? And what is the exact mechanism that mediates the therapeutic effects? It is therefore important to start with a proper characterization of (biased) H_4R signal transduction pathways.
In chapter 3 we presented several $G\alpha_i$ and β-arrestin 2 biased ligands that will be interesting tool compounds to specifically target these downstream signaling routes. In addition, in chapter 5 we discovered fragments that target several histamine receptors with a variety of functional efficacies. Consequently, we can select lead fragments with desired pharmacologies to develop multi-receptor drugs. It is for instance suggested that targeting multiple histamine receptors (i.e. H_1R and H_4R) works synergistically in the treatment of inflammatory conditions[177].

FINAL CONCLUSIONS

In this thesis we set ourselves three goals to better understand and analyse the molecular pharmacology (i.e. ligand binding, biased signalling) of the human histamine H_4 receptor.
We validated the H_4R binding mode of 2-aminopyrimidines by developing an H_4R ligand (VUF14480) that was rationally designed to irreversibly interact with $C^{3.36}$ in H_4R. VUF14480 partially activated the H_4R, which led us to hypothesize that H_4R activation is dependent on the disruption of polar network(s) (mediated by water molecules) within the receptor (Chapter 2 and 4).
Chemically diverse H_4R ligands were evaluated for their ability to induce $G\alpha_i$ protein activation and β-arrestin recruitment. Several biased H_4R ligands were discovered and we could hint at the structural determinants of both indolecarboxamide ligands and H_4R involved in biased signalling (Chapter 3, 4, 5). The biased H_4R compound selection will be useful in future research to dissect the distinct H_4R downstream pathways after biased activation. Further investigation into the structural requirements for β-arrestin signalling identified subtle conformational changes that could play a role in biased H_4R signalling. We hypothesize therefore that β-arrestin activation depends less on polar interactions than G protein activation.
The assays used in the here described studies were optimized and miniaturized to a homogenous 384 well format without losing assay quality. Their relatively high throughput format allows for a quick and broad fragment/ligand/metabolite screen, which will form the basis for future medicinal chemistry programs.