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Title page

A structural insight into the reorientation of transmembrane domains 3 and 5 during family A GPCR activation

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Running title page

a) Running title: Role of TMs 3 and 5 in receptor activation

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Discussion: 1153 words

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d) List of abbreviations:

G protein-coupled receptors, GPCRs; H_1 receptor, H_1R; wild-type, WT; transmembrane, TM; *trans*, *t*; *gauche+*, *g+*; *gauche-*, *g-*

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Abstract

Rearrangement of transmembrane domains (TMs) 3 and 5 following agonist binding, is necessary for stabilization of the active state of Class A G protein-coupled receptors (GPCRs). Using site-directed mutagenesis and functional assays, we provide the first evidence that the TAS(I/V) sequence motif at positions 3.37-3.40, highly conserved in aminergic receptors, plays a key role in the activation of the histamine H_1 receptor. By combining these data with structural information from X-ray crystallography and computational modeling, we suggest that $T^{3.37}$ interacts with TM5 stabilizing the inactive state of the receptor, while the hydrophobic side chain at position 3.40, highly conserved in the whole Class A GPCR family, facilitates the reorientation of TM5. We propose that the structural change of TM5 during the process of GPCR activation involves a local $P^{5.50}$-induced unwinding of the helix, acting as a hinge, and the highly conserved hydrophobic $I^{3.40}$ side chain, acting as a pivot.

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Introduction

G protein-coupled receptors (GPCRs) transduce sensory signals of external origin such as photons, odors or pheromones and endogenous signals, including biogenic amines, (neuro)peptides, proteases, glycoprotein hormones and ions, to the cytoplasmic side of the cell membrane (Kristiansen, 2004). The mechanism by which binding of these highly diverse chemical signals triggers a set of conformational rearrangements of the transmembrane (TM) segments near the G-protein binding domains remains largely unknown. Nevertheless, comparison of the structure of inactive rhodopsin (Li et al., 2004) with the recent crystal structure of the ligand-free opsin (Park et al., 2008), which contains several distinctive features of the presumed active state, leads to conclude that during the process of GPCR activation, among others changes, TM3 rotates clockwise (viewed from the intracellular side), the intracellular part of TM6 tilts outwards by 6-7 Å, TM5 approaches TM6, and $R^{3.50}$ (see Experimental Procedures for the general numbering scheme) within the (D/E)R(Y/W) motif in TM3 adopts an extended conformation pointing towards the protein core (Park et al., 2008). Notably, as revealed in the original publication of the opsin crystal structure, these conformational changes disrupt the ionic interaction between $R^{3.50}$ with negatively charged side chains at positions 3.49 and 6.30 (see also Smit et al. 2007, and Rosenbaum et al. 2009 for reviews of conserved amino acids involved in GPCR activation) and facilitate the interactions between the highly conserved $Y^{5.58}$ and $K^{5.66}$ in TM5 and $R^{3.50}$ in TM3 and $E^{6.30}$ in TM6, respectively. These structural changes strongly suggest that both TMs 3 and 5 play a central role in stabilizing the active state of GPCRs and, therefore, in the process of GPCR activation.

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In this study, we have combined the latest structural insights in GPCR structure, computational modeling and site-directed mutagenesis to study the role of the TAS(I/V) motif at positions 3.37-3.40 in TM3, highly conserved in aminergic receptors, in the process of receptor activation. Our results suggest that $T^{3.37}$ interacts with TM5 in the inactive state of the receptor. In addition, we provide the first evidence that the hydrophobic side chain at position 3.40, highly conserved in the whole Class A of GPCRs, plays a key role in activation. Mutation of $I^{3.40}$ to either Ala or Gly, i.e. removing the bulky side chain at this position, abolishes the constitutive activity of the histamine H_1 receptor (H_1R), the effect of constitutive-activity increasing mutations, as well as the histamine-induced receptor activation.

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Materials and Methods

Materials

Gifts of mianserin hydrochloride (Organon NV, The Netherlands), pcDEF$_3$ (Dr. J. Langer, Goldman et al., 1996) and of the cDNA encoding the human histamine H_1R (Dr. H. Fukui, Fukui et al., 1994) are greatly acknowledged. pNF-κB-Luc was obtained from Stratagene (USA), ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, mepyramine (pyrilamine maleate) and polyethylenemine were purchased from Sigma Chemical Company (USA). D-luciferin was obtained from Duchefa Biochemie BV (The Netherlands), glycerol from Riedel-de-Haen (Germany), and Triton X-100 from Fluka (Switzerland). Cell culture media, penicillin and streptomycin were obtained from Invitrogen Corporation (UK). Fetal bovine serum was obtained from Intergro B.V. (The Netherlands). Cell culture plastics were obtained from Corning Costar (USA). [^{3}H]mepyramine (30 Ci/mmol) was purchased-from ICN Biomedicals BV (The Netherlands).

Cell culture and transfection

COS-7 African green monkey kidney cells were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) containing 50 IU/ml penicillin, 50 µg/ml streptomycin and 5% (v/v) fetal bovine serum. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described (Bakker et al., 2001).

Site-directed mutagenesis

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Single point mutant human histamine H_1Rs $T^{3.37}A$, $T^{3.37}E$, $I^{3.40}A$ and $I^{3.40}G$ and double mutant human histamine H_1Rs $S^{3.36}T/I^{3.40}A$, $S^{3.36}T/I^{3.40}G$, $I^{6.40}K/I^{3.40}A$ and $I^{6.40}S/I^{3.40}A$ were created using a PCR-based mutagenesis approach. All constructs were sub-cloned into the expression vector $pcDEF_3$ and verified by DNA sequencing.

Reporter-gene assay

Reporter-gene assays were performed essentially as described previously (Bakker et al., 2001). In brief, cells transiently co-transfected with pNF-κB-Luc (125 μg/1.10^7 cells) and $pcDEF_3$ encoding WT or mutant H_1Rs (25 μg/1.10^7 cells) were seeded in 96 well white plates (Costar) in serum free culture medium and incubated with drugs. After 48 hrs, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μl/well luciferase assay reagent (0.83 mM ATP. 0.83 mM d-luciferin, 18.7 mM $MgCl_2$, 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 30 min, luminescence was measured for 3 sec/well in a Victor^2 plate reader (PerkinElmer, USA). Structural rearrangements during GPCR activation probably occur on a millisecond time scale and, therefore, this assay cannot assess the influence of the mutations on these fast processes. However, we show that specific mutations disrupt receptor activation, probably by stabilizing non-functional conformations. We are able to detect these types of disruptions by measuring the changes in receptor signaling in the reporter-gene assay. This assay has been shown to be comparable to the classical G_q-linked generation of inositol phosphates (Bakker et al., 2001).

Histamine H_1R binding studies

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Histamine H_1R binding studies were performed essentially as described previously (Bakker et al., 2001). In brief, transfected COS-7 cells used for radioligand binding studies were harvested after 48hrs and homogenized in 50 mM of ice-cold Na_2/K-phosphate buffer (pH 7.4) (binding buffer). The COS-7 cell homogenates were incubated for 30 min at 30 °C in binding buffer in 200 µl with 3 nM [^{3}H]mepyramine. Non-specific binding was determined in the presence of 1 µM mianserin. The incubations were stopped by rapid dilution with 3 ml of ice-cold binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml of binding buffer and the radioactivity retained on the filters was measured by liquid scintillation counting.

Analytical methods

Protein concentrations were determined according to Bradford (Bradford, 1976), using BSA as a standard. Binding and functional data were evaluated by a non-linear, least squares curve-fitting procedure using GraphPad Prism 4® (GraphPad Software, Inc., San Diego, CA, USA).

Computational model of the histamine H_1R

A model of the TM domains 1-7 of the histamine H_1R was built by homology modeling using the crystal structure of the β_2-adrenergic receptor (PDB code 2RH1) (Rosenbaum et al., 2007) as template. Building this homology model was straightforward except for the highly conserved $NPxxYx_{n=5,6}F$ motif at the junction between TM7 and the intracellular helix 8. Notably, this junction is one residue shorter in the β_2-adrenergic receptor (n=5) than in rhodopsin (n=6). As a result, $Y^{7.53}$ is pointing towards TM2 in rhodopsin (Li et al., 2004) and towards TM6 in the β_2-adrenergic receptor (Rosenbaum et al., 2007). The fact that the histamine H_1R contains six amino acids (n=6) between both aromatic side chains led us to model the junction between TM7 and

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helix 8 as in rhodopsin (PDB code 1GZM). Modeller 9v1 (Marti-Renom et al., 2000) was used to add intracellular loops IL1-2 and extracellular loops EL1-3 using the structure of the β_2-adrenergic receptor as template. Internal water molecules 506, 519, 528, 529, 532, 534, 537, 543, 546, and 548 that mediate a number of key interhelical interactions (Rosenbaum et al., 2007) and are probably conserved in family A GPCRs (Pardo et al., 2007) were explicitly included in the model.

Distortion of TM5

The program HELANAL (Bansal et al., 2000) was used to calculate the residue-residue unit twist angle of TM5, a structural parameter that describes local helical uniformity. This parameter is interpreted as follows: an ideal α-helix, with approximately 3.6 residues per turn, has a twist angle of approximately 100º (360º/3.6); a closed helical segment, with <3.6 residues per turn, possesses a twist >100º; whereas an open helical segment, with >3.6 residues per turn, possesses a twist <100º.

Nomenclature of side chain conformation

The side chain conformations have been categorized into *gauche*- (*g*-: $0° < \chi_1 < 120°$), *trans* (*t*: $120° < \chi_1 < 240°$), or *gauche*+ (*g*+: $240° < \chi_1 < 360°$) depending on the value of the χ_1 torsional angle.

Numbering Scheme of GPCRs

Residues are identified by the general numbering scheme of Ballesteros and Weinstein (1995) that allows easy comparison among residues in the 7TM segments of different receptors.

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Sequence analysis

Family-specific analyses of GPCR sequence conservation were performed using the GMoS program, freely available at http://lmc.uab.cat/gmos.

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Results

A proline-induced unwinding in the structure of TM5 of Class A GPCRs

GPCRs possess a highly conserved $P^{5.50}$ in TM5, present in 77% of the rhodopsin-like sequences, absent only in melanocortin, glycoprotein hormone, lysosphingolipid, prostanoid, and cannabinoid receptors. Usually, in Pro-containing α-helices, the steric clash between the pyrrolidine ring of Pro and the backbone carbonyl oxygen in the preceding turn induces a bend angle of approximately 20º in the helical structure (Deupi et al., 2004). However, a detailed analysis of the structures of bovine (Li et al., 2004) and squid (Murakami and Kouyama, 2008) rhodopsin, adenosine A_{2A} receptor (Jaakola et al., 2008), and the β_1- (Warne et al., 2008) and β_2- (Rosenbaum et al., 2007) adrenergic receptors has allowed us to detect and quantify a peculiar distortion of TM5 in which a local opening of the helix (>3.6 residues/turn, twist < 100º, see Experimental Procedures) at the 5.45-5.48 turn (Fig. 1B) partially removes the steric clash between the pyrrolidine ring of $P^{5.50}$ and the backbone carbonyl oxygen at position 5.46 (i-4 in Figs. 1C-1H). This local opening of TM5 (*proline-unwinding*, in contrast to *proline-kink*) also modifies the relative orientation of the side chains at the extracellular side, including residues involved in neurotransmitter binding such as 5.42, 5.43, and 5.46 (Ballesteros et al., 2001b; Deupi et al., 2007). Other membrane proteins also feature this type of helical wide turns (Riek et al., 2008).

Role of TM3 in stabilizing the proline-unwinding of TM5

The analysis of the currently available GPCR crystal structures in the present study allows us to propose a common mechanism by which this unusual conformation of TM5 is stabilized. In

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bovine rhodopsin, the backbone carbonyl oxygen at position 5.46 is stabilized by a hydrogen bond interaction with $E^{3.37}$ (at the inter-atomic distance of 2.8Å) and a van der Waals interaction with $L^{3.40}$ (3.2Å) (Figs. 1C and 2A) (Deupi et al., 2007; Li et al., 2004). Adenosine A_{2A} receptor preserves similar interactions through $Q^{3.37}$ (3.2Å) and $I^{3.40}$ (3.9Å) (Fig. 1G) (Jaakola et al., 2008). Interestingly, squid rhodopsin replaces $E^{3.37}$ of bovine rhodopsin by Phe but presents a discrete water molecule linking the backbone carbonyls at positions 3.37 (3.0Å) and 5.46 (3.3Å) (Murakami and Kouyama, 2008), while maintaining the van der Waals interaction between the hydrophobic $I^{3.40}$ and the carbonyl oxygen at position 5.46 (3.2Å) (Fig. 1D). Notably, the recent X-ray structure of opsin (Park et al., 2008) shows that during the process of rhodopsin activation the hydrogen bond interaction between $E^{3.37}$ and the backbone carbonyl (4.9Å) is disrupted, as previously suggested by NMR measurements of rhodopsin containing ^{13}C-labeled histidine (Patel et al., 2005), while $L^{3.40}$ has moved slightly away (3.5Å) (Figs. 1F and 2B). This effect correlates with the minor, but significant, clockwise rotation (viewed from the intracellular side) of TM3 during the process of receptor activation (see Discussion). The release of the strong packing of $L^{3.40}$ with the 5.45-5.48 turn does not result in a change in the local opening of TM5 (Fig. 1B), but in a localized decrease in the bend around $P^{5.50}$ (Fig. 1A). In the β_1- (Warne et al., 2008) and β_2- (Rosenbaum et al., 2007) adrenergic receptors, $I^{3.40}$ also stabilizes the carbonyl oxygen at position 5.46, similarly to adenosine and opsin receptors, whereas, in this case, the shorter $T^{3.37}$ (compared to E or Q) interacts with the side chain, rather than with the backbone carbonyl, of $S^{5.46}$ (Figs. 1E, 1H and 2C).

To investigate the role of the amino acids at positions 3.37 and 3.40 in GPCR activation we have created and studied several histamine H_1 receptor (H_1R) mutants. We have previously used the H_1R as a model system for the study of Class A GPCR activation (Bakker et al., 2008). The H_1R

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belongs to the aminergic subfamily of Class A GPCRs, and like the β_1- and β_2-adrenergic receptors features $T^{3.37}$ and $I^{3.40}$ in TM3. Moreover, the H_1R contains a polar $N^{5.46}$ amino acid at position 5.46, which is involved in histamine binding (Leurs et al., 1994), linking this GPCR subdomain directly to the agonist binding site.

Influence of $T^{3.37}$ and $I^{3.40}$ in agonist-induced activation of the human histamine H_1R

In order to test the role of the amino acids at positions 3.37 and 3.40 we engineered mutants in which $T^{3.37}$ of the histamine H_1R was substituted by either Ala ($T^{3.37}A$) to remove the hydrogen bonding capability at this position, or Glu ($T^{3.37}E$) to mimic the bovine rhodopsin sequence, and $I^{3.40}$ was replaced by either Ala ($I^{3.40}A$) or Gly ($I^{3.40}G$) removing the bulky hydrophobic side chain and, thus, the van der Waals interaction with the carbonyl group at position 5.46. Fig. 3D shows the environment of $T^{3.37}$ in the β_2-based molecular model of the histamine H_1R (see Experimental Procedures). $T^{3.37}$ forms a hydrogen bond with $N^{5.46}$, the binding partner for the imidazole ring of histamine (Leurs et al., 1994), as observed for the $T^{3.37}$•••$S^{5.46}$ interaction in the structure of the β_2-adrenergic receptor (Fig. 1H). Wild type, $T^{3.37}A$ and $T^{3.37}E$ mutants H_1Rs were well expressed in COS-7 cells at about 10 pmol/mg protein as determined by radioligand binding analysis (Table 1). The function of the WT H_1R was evaluated in a NF-κB reporter-gene assay as previously reported (Bakker et al., 2008; Jongejan et al., 2005), and the activation of NF-κB was increased up to 10 fold (E_{max} = 1002 ± 34%) when stimulated with histamine (Fig. 3A and 3B). The fact that the functional potency of histamine at WT H_1R exceeds its binding affinity is well in accordance with previously published data (Bakker et al., 2008; Jongejan et al., 2005). Removal of the interaction of $T^{3.37}$ with $N^{5.46}$, by mutating residue $T^{3.37}$ to Ala resulted in a 0.6 log unit decrease of the binding affinity of histamine (pK_i of 5.3 for the WT H_1R vs. 4.7 for the

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$T^{3.37}A$ H_1R) (Table 1) as measured by [^{3}H]mepyramine displacement. A lower constitutive signaling of the $T^{3.37}A$ H_1R mutant compared to that of WT H_1R was observed (Figs. 3A and 3B). However, the basal signaling could still be increased by histamine albeit at higher concentrations (pEC_{50}=4.5±0.2) (Table 1) (Supplemental Figure 1). Mutation of residue $T^{3.37}$ to Glu (i.e. making this region similar to bovine rhodopsin) did not influence the binding affinity of histamine (Table 1), possibly because $E^{3.37}$ maintains both the interaction with $N^{5.46}$ (Fig. 3E), as in the WT H_1R (Fig. 3D), and with the carbonyl group at position 5.46, as in bovine rhodopsin (Fig. 1C). Notably, the $T^{3.37}E$ mutant H_1R hardly shows any constitutive activity (Figs. 3A and 3B). Moreover, the $T^{3.37}E$ mutant H_1R is activated very poorly by histamine (pEC_{50}>3.2). Thus, we propose that the additional constraint between $E^{3.37}$ and the backbone carbonyl at position 5.46, introduced in the $T^{3.37}E$ mutation, impedes the activation of the mutant H_1R by locking a local structure that cannot be overcome by the agonist histamine.

Removal of the bulky hydrophobic $I^{3.40}$ side chain by substitution to Ala or Gly also results in significant effects on both the constitutive activity of the receptor and the process of histamine-induced receptor activation (Figs. 3A and 3C). Importantly, these effects are not due to a loss of binding affinity for histamine. As shown in Table 1 both $I^{3.40}A$ and $I^{3.40}G$ mutant H_1Rs are well expressed in COS-7 cells and bind histamine with comparable affinity compared to WT H_1R. The fact that removal of the $I^{3.40}$ side chain abolished both the constitutive and histamine-induced activity strongly suggests that this hydrophobic side chain plays a key role in the mechanism of activation. Based on these results and on the recent insights in GPCR structure (Park et al., 2008; Scheerer et al., 2008), we propose that the hydrophobic side chain at position 3.40, which is strongly packed against TM5 (Fig. 2), acts as a pivot and the $Pro^{5.50}$-induced unwinding acts as a hinge in the crucial reorientation of TM5 upon activation, which ultimately facilitates the

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interactions of $Y^{5.58}$ and $K^{5.66}$ with $R^{3.50}$ and $E^{6.30}$, respectively (Fig. 2). Notably, mutations at position 3.40 in rhodopsin are related to retinitis pigmentosa, poor retinal binding, misfolding, reduced expression, decreased time in the MII state, and reduced transducin activation (Madabushi et al., 2004).

To substantiate this hypothesis, we performed two sets of additional experiments. We combined activating point mutations leading to agonist-independent constitutive activity with the inactivating $I^{3.40}$A/G mutation with the aim to assess their compensatory consequences on receptor function. We have chosen the previously reported $S^{3.36}$T mutation (Jongejan et al., 2005) in the ligand binding site and the $I^{6.40}$K/S mutations (Bakker et al., 2008) near the cytoplasmic site, both leading to a large increase in constitutive activity (see Discussion). The $S^{3.36}$T mutation induces the transition of $W^{6.48}$ toward TM5 (Jongejan et al., 2005), considered to be the initial stage of the activation process (see below), as observed in the electron microscopy density map of metarhodopsin I (Ruprecht et al., 2004) and in solid-state NMR measurements of metarhodopsin II (Crocker et al., 2006). Thus, we combined the activating $S^{3.36}$T point mutation with the inactivating $I^{3.40}$A/G mutation to generate the $S^{3.36}$T/$I^{3.40}$A and $S^{3.36}$T/$I^{3.40}$G double mutant H_1Rs. The double mutant H_1Rs are well expressed in COS-7 cells and have similar affinity for histamine compared to wild-type H_1R (Table 1). Interestingly, both double mutants show lack of constitutive activity and cannot be activated by histamine, resembling the single $I^{3.40}$A/G mutant H_1R (Fig. 4). On the other hand, the $I^{6.40}$S and $I^{6.40}$K mutations induce constitutive receptor activation by triggering the conformational change of $N^{7.49}$ toward $D^{2.50}$ (Bakker et al., 2008; Urizar et al., 2005). The $I^{6.40}$S /$I^{3.40}$A and $I^{6.40}$K /$I^{3.40}$A double mutant H_1Rs show similar affinities for histamine binding (Table 1), but lack constitutive activity and cannot

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be activated by histamine (Fig. 4). These data reinforce the importance of $I^{3.40}$ in the process of receptor activation.

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Discussion

A number of studies have provided convincing evidence that GPCRs co-exist in different conformations (see Kobilka and Deupi, 2007 and references therein). GPCRs are maintained within an ensemble of inactive conformations through non-covalent interactions between side chains, mostly located in the TM segments (Smit et al., 2007; Rosenbaum et al., 2009) but also at the extracellular surface (Bokoch et al., 2010). Disruption of these stabilizing interactions results in gain-of-function mutations. An evolutionary trace analysis has led to the identification of a putative common signal transduction process for Class A GPCRs, where key residues are grouped into a network of interactions extending from a trigger region near the binding pocket to a coupling region in the cytoplasmic region, connected by an intermediate linking core (Madabushi et al., 2004). This analysis detected $L^{3.40}$ of rhodopsin in the trigger region as a structurally and/or functionally important residue.

The fact that the histamine H_1R shows considerable agonist-independent, constitutive signaling, which can be strongly reduced by the inverse agonist mepyramine (Bakker et al., 2001), provides an unique opportunity to approach the mechanism of receptor activation. In this study, we employed site-directed mutagenesis of the H_1R to study the proposed role of the hydrophobic side chain at position 3.40, highly conserved in the whole Class A of GPCRs, in GPCR activation. Mutation of $I^{3.40}$ to either Ala or Gly, removing the bulky side chain, abolished the constitutive activity of the receptor, the effect of constitutive-activity increasing mutations, and the histamine-induced receptor activation, but not the binding of histamine. These data strongly suggest that $I^{3.40}$ is not involved in the initial binding step, but participates in the subsequent signal propagation upon histamine binding. In the inactive state (Figs. 2A and 2C) the

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hydrophobic side chain at position 3.40 (green surface) is located between the pyrrolidine ring of $P^{5.50}$ and the carbonyl oxygen at position 5.46, inducing or stabilizing the local opening of TM5 (Fig. 1B). However, the molecular mechanism by which this residue facilitates receptor activation is not straightforward. The structure of opsin (Park et al., 2008) and previous substituted-cysteine accessibility studies in the β_2-adrenergic receptor (Gether et al., 1997) have shown a clockwise rotation (viewed from the intracellular side) of TM3 during the process of receptor activation. Rotation of TM3 moves the bulky side chain at position 3.40, located between $P^{5.50}$ and the 5.46 carbonyl, away from these moieties (Fig. 2B), resulting in a localized decrease of the bend around $P^{5.50}$ (Fig. 1A).

Fluorescence spectroscopy experiments used to monitor agonist-induced conformational changes in the β_2-adrenergic receptor provide evidence of a sequential binding model through discrete intermediate conformational states (Swaminath et al., 2004). In an initial step agonist binding induces relocation of the extracellular side of TM5 to facilitate binding of the hydroxyl groups of the catechol moiety of β_2-adrenergic receptor agonists (Kobilka, 2004). We propose that this movement is transduced as a set of structural rearrangements toward the intracellular side of TM5 with the assistance of $I^{3.40}$ acting as a pivot, ultimately enabling the interactions of $Y^{5.58}$ with $R^{3.50}$ and $K^{5.66}$ with $E^{6.30}$ in the cytoplasmic side of the receptor (Fig. 5, right arrow). In support of this hypothesis, the $T^{3.37}E$ mutation in the H_1R, which adds an additional constraint between TMs 3 and 5 (Fig. 3E), prevents the transmission of the activation signal through TM5 (Fig. 3B), but does not affect the binding of histamine.

In addition to this activation pathway through TM5, amino acids located in TMs 3 and 6 also participate in other routes of receptor activation (Fig. 5, top/left arrow). For instance, agonist

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binding triggers the $W^{6.48}$ rotamer toggle switch from the inactive *g+* (pointing towards TM7) to the active *t* (towards the binding pocket) conformation (Crocker et al., 2006; Holst et al., 2010; Ruprecht et al., 2004; Shi et al., 2002), through the formation of specific hydrogen bonds (Lopez-Rodriguez et al., 2005). Notably, the rotamer toggle switch of $W^{6.48}$ occurs in a concerted manner with the side chain at position 3.36 from the inactive *t* (towards the binding pocket) to the active *g+* (towards TM7) conformation (Jongejan et al., 2005). In order to assess whether these pathways of receptor activation are independent or not, we have combined the inactivating $I^{3.40}$A/G mutations (Figs. 4) with the activating $S^{3.36}$T point mutation, which is also located in the trigger region and which leads to agonist-independent constitutive activity (Jongejan et al., 2005). The absence of both constitutive activity and histamine-induced activation in these double mutant receptors, despite normal histamine binding, led us to conclude that the movement of TM5 (via the $I^{3.40}$ pivot) is essential to stabilize the activation pathway by means of the $W^{6.48}$/$S^{3.36}$ rotamer toggle switch.

The signal triggered by the $W^{6.48}$/$S^{3.36}$ toggle switch is transmitted towards intracellular microdomains (Rosenbaum et al., 2009; Smit et al., 2007). In particular, $N^{7.49}$ and $Y^{7.53}$ (in the NPxxY motif of TM7) undergo conformational changes (Park et al., 2008; Urizar et al., 2005), ultimately leading to the disruption of the ionic interaction between $R^{3.50}$ and the adjacent D/$E^{3.49}$ (the DRY motif of TM3) (Scheer et al., 1996), and an additional D/$E^{6.30}$ near the cytoplasmic end of TM6 (Ballesteros et al., 2001a) (Fig. 5, bottom/left arrow). Disruption of these ionic interactions permits $R^{3.50}$ to adopt an extended conformation (Bakker et al., 2008; Park et al., 2008), pointing toward the protein core and to interact with the highly conserved $Y^{5.58}$ and $Y^{7.53}$ in TMs 5 and 7 (Park et al., 2008). These conformational changes open a cavity between TMs 3, 5 and 6 where the G protein can bind (Scheerer et al., 2008). We have previously shown that

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mutation of the highly conserved hydrophobic side chain at position 6.40 to either Glu, Gly, Ala, Arg, Lys, or Ser results in highly constitutively active receptors for which almost no additional histamine-induced receptor activation can be detected (Bakker et al., 2008). These substitutions at position 6.40 appear to modify the conformation of the key $N^{7.49}$, $Y^{7.53}$ and $R^{3.50}$ side chains, located in the coupling region near the G protein binding site (Bakker et al., 2008). Notably, adding the $I^{3.40}A$ mutation, in the trigger region, to the constitutively active $I^{6.40}S/K$ mutant receptors decreases constitutive activity and impedes histamine-induced receptor activation (Fig. 4). These results suggest a key role of $I^{3.40}$ in transmitting the activating signal from the binding pocket to the cytoplasmic side and in stabilizing the conformational changes of TM5 at the intracellular side of the receptor, probably by means of $Y^{5.58}$.

Triggering specific pathways along the activation process might be an explanation for the observed collateral efficacy of GPCR agonists. Our data provide further insight in the molecular mechanisms of GPCR activation and we propose that one of such additional pathways consists in the agonist-induced relocation of the extracellular side of TM5 towards the transmembrane bundle. The activation-signal is then further propagated towards the cytoplasmic region through the fulcrum/lever mechanism described above, in which $P^{5.50}$ acts as a hinge and the highly conserved hydrophobic $I^{3.40}$ side chain acts as a pivot.

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Authorship contribution

Participated in research design: Sansuk, Bakker, Jongejan, Pardo and Leurs

Conducted experiments: Sansuk, Torrecillas and Nijmeijer,

Contributed new reagents or analytic tools: -

Performed data analysis: Sansuk, Deupi, Torrecillas and Nijmeijer,

Wrote or contributed to the writing of the manuscript: Sansuk, Deupi, Bakker, Pardo and Leurs

Others: -

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Footnotes

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Figure legends

Fig. 1 Comparison of the local opening of transmembrane helix 5 in bovine (PDB code 1GZM) and squid (2Z73) rhodopsin, opsin (3CAP), adenosine A_{2A} (3EML) and β_1- (2R4R) and β_2- (2RH1) adrenergic receptors.

(A, B) Evolution of local bend (A) and helical twist (B) angles (°, see Experimental Procedures) along TM5 in the crystal structures of the β_2-adrenergic receptor (blue), bovine rhodopsin (black) and opsin (red). Residue numbers refer to the first residue in each turn; i.e. the peak of the helical distortion appears in the turn 5.45-5.48, labeled as 5.45 in the graphic. (C-H) Detailed view of the interface between TMs 3 (dark red) and 5 (green) in bovine (C) and squid (D) rhodopsin, opsin (F), adenosine A_{2A} (G) and β_1– (E) and β_2- (H) adrenergic receptors.

Fig. 2 Packing of transmembrane helix 3 against $P^{5.50}$ and network of ionic interactions at the cytoplasmic domain.

Packing of the hydrophobic and bulky side chain at position 3.40 with $P^{5.50}$ (green spheres) and network of interactions of $R^{3.50}$, $Y^{5.58}$, $K^{5.66}$ and $E^{6.30}$ in TMs 3 (dark red), 5 (green) and 6 (yellow) in bovine rhodopsin (A), opsin (B), and the β_2-adrenergic receptor (C) in views parallel (top) and perpendicular (bottom) to the membrane.

Fig. 3 Influence of $T^{3.37}$ and $I^{3.40}$ on the agonist-induced activation of the histamine H_1 receptor. NF-κB activation modulated by histamine in COS-7 cells transiently transfected with wild type (WT), $T^{3.37}$, $I^{3.40}$, $S^{3.36}$and empty vector ($pcDEF_3$). All concentration response curves were constructed with GraphPad Prism version 4.0 ($N \geq 2$, each performed in triplicate).

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(A) Basal activity (black bars) and after stimulation with 10^{-4} M histamine (grey bars). Results are normalized to the basal activity of WT which is set to 100%. (B) Representative concentration response curves of histamine for WT (●), $T^{3.37}A$ (○), $T^{3.37}E$ (■), and $pcDEF_3$ (□) (C) Representative concentration response curves of histamine for WT (●), $I^{3.40}A$ (○), $I^{3.40}G$ (■), and $pcDEF_3$ (□) (D, E) Detailed view of the interface between TMs 3 and 5 in the molecular model of WT (D) and mutant $T^{3.37}E$ (E) histamine H_1R.

Fig. 4 Influence of double mutants $S^{3.36}T/I^{3.40}G$, A and $I^{6.40}K,S/I^{3.40}A$ on the agonist-induced activation of the histamine H_1 receptor. NF-κB activation modulated by histamine in COS-7 cells transiently transfected with wild type (WT), double H_1R mutants and empty vector ($pcDEF_3$). All concentration response curves were constructed with GraphPad Prism version 4.0 (N≥2, each performed in triplicate)

(A) Basal activity (black bars) and after stimulation with 10^{-4} M histamine (grey bars). Results are normalized to the basal activity of WT which is set to 100%. (B) Representative concentration response curves of histamine for WT (●), $S^{3.36}T$ (○), $S^{3.36}T/I^{3.40}G$ (■), $S^{3.36}T/I^{3.40}A$ (□), $I^{6.40}K$ (▲), $I^{6.40}S$ (Δ), $I^{6.40}K/I^{3.40}A$ (♦), $I^{6.40}S/I^{3.40}A$ (◊) and $pcDEF_3$ (✳)

Fig. 5 Pathways of activation in Class A GPCRs.

Agonist (shown as ball-and-sticks) binding leads to conformational changes at the extracellular domain that are transmitted (arrows) towards the cytoplasmic domain of the receptor. The right arrow represents how the interaction of agonists with the extracellular side of TM5 is transduced towards the intracellular side with the assistance of $I^{3.40}$ (shown as spheres) and the $Pro^{5.50}$-induced unwinding of TM5, facilitating the interaction of $Y^{5.58}$ with $R^{3.50}$ in the active state

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(yellow sticks). Agonist binding may also trigger the $W^{6.48}/S^{3.36}$ rotamer toggle switch (top/left arrow). This agonist-induced signal is transmitted through a conserved water cluster (red spheres) towards intracellular microdomains like $N^{7.49}$ and $Y^{7.53}$ of the NPxxY motif (bottom/left arrow), facilitating the interaction of $Y^{7.53}$ with $R^{3.50}$ in the active state (yellow sticks). Disruption of the ionic interactions between $R^{3.50}$ and the adjacent $D^{3.49}$ (the DRY motif of TM3) and an additional $E^{6.30}$ facilitates the movement of TM6 (not shown), opening a cavity in which the G protein can bind (not shown). Side chains of amino acids modeled in inactive conformations are depicted in white, in their putative active conformations in yellow, and the side chains of $S^{3.36}$, $I^{3.40}$, and $I^{6.40}$, which are discussed in this manuscript, are depicted as spheres.

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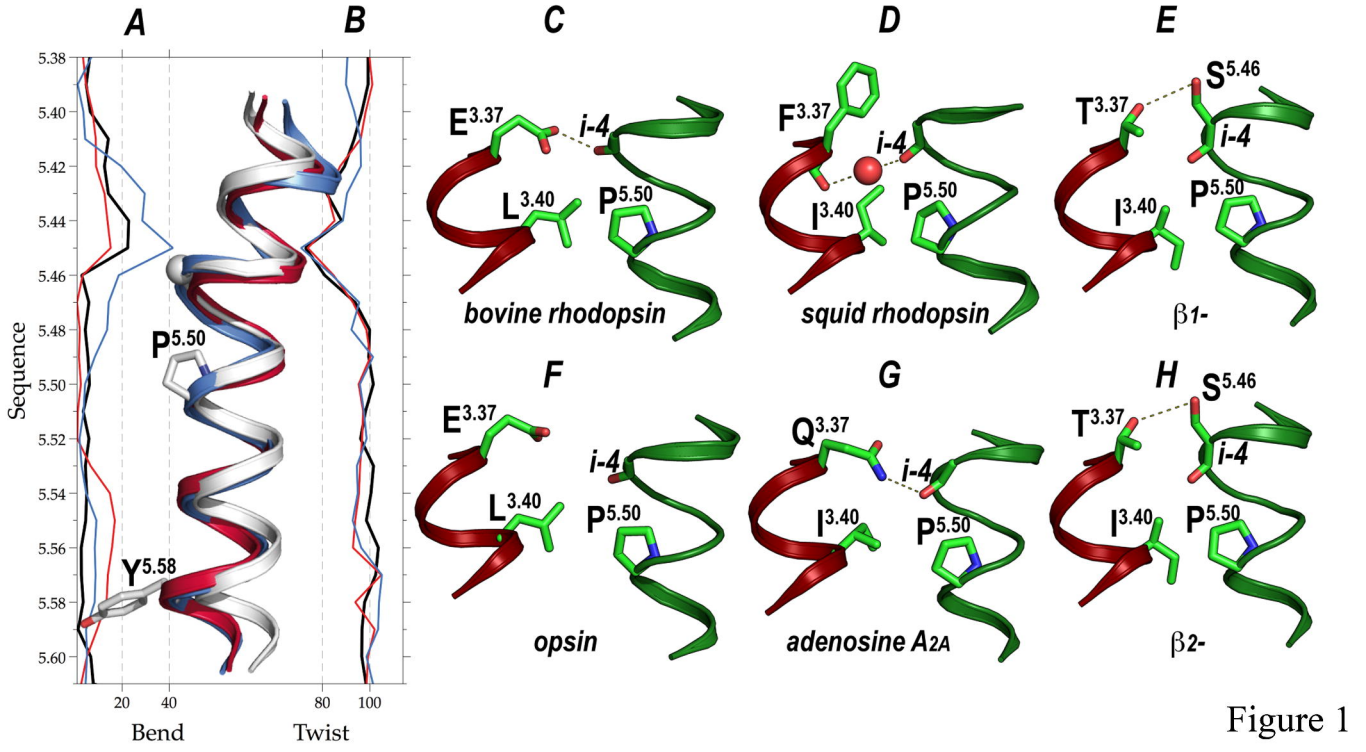
Table 1. Pharmacological characteristics of hH_1R wild type (WT) and hH_1R mutants transiently expressed in COS-7 cells. The potency (pEC_{50}) of histamine was measured by NF-κB reporter gene assay. Affinities of [^{3}H]mepyramine (K_D) and H_1R expression levels (B_{max}) are determined by saturation radioligand binding assays. Binding affinity values of H_1R ligands are determined by [^{3}H]mepyramine displacement. Data were calculated as the mean ± S.E.M. (n) is number of experiments, each performed in triplicate.

Receptor	**[^{3}H]mepyramine**		**Histamine**	
	K_D (nM) ± S.E.M. (n)	**B_{max} (pmol/mg protein) ± S.E.M. (n)**	**pK_i ± S.E.M. (n)**	**pEC_{50} ± S.E.M. (n)**
hH_1R WT	1.5 ± 0.2 (7)	14.8 ± 4.6 (7)	5.3 ± 0.1 (4)	6.5 ± 0.1 (5)
$T^{3.37}A$	5.1, 4.5	10.6, 6.5	4.7 ± 0.1 (4)	4.5 ± 0.2 (3)
$T^{3.37}E$	6.1, 6.2	9.6, 15.9	5.1 ± 0.1 (4)	> 3.2 (3)
$I^{3.40}A$	10.1 ± 0.3 (3)	9.4 ± 5.1 (3)	5.3 ± 0.2 (4)	> 3.4 (3)
$I^{3.40}G$	7.3, 7.6	7.6, 9.8	5.0 ± 0.2 (3)	> 3.8 (2)
$S^{3.36}T + I^{3.40}A$	7.8 ± 0.3 (4)	10.4 ± 5.3 (4)	5.4 ± 0.1 (3)	> 3.7 (2)
$S^{3.36}T + I^{3.40}G$	3.4 ± 0.2 (3)	13.4 ± 5.0 (3)	5.0 ± 0.1 (3)	> 3.9 (2)
$I^{6.40}K + I^{3.40}A$	2.7, 2.1	10.7, 6.5	4.9, 5.4	4.6, 4.5
$I^{6.40}S + I^{3.40}A$	2.2, 1.6	8.7, 5.1	4.6, 5.0	>3.6 (2)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D
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G
H
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5.58
5.60
P5.50
Y5.58
20
40
80
100
Bend
Twist
E3.37
i-4
L3.40
P5.50
bovine rhodopsin
F3.37
i-4
I3.40
P5.50
squid rhodopsin
T3.37
S5.46
i-4
I3.40
P5.50
β1-
E3.37
i-4
L3.40
P5.50
opsin
Q3.37
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adenosine A2A
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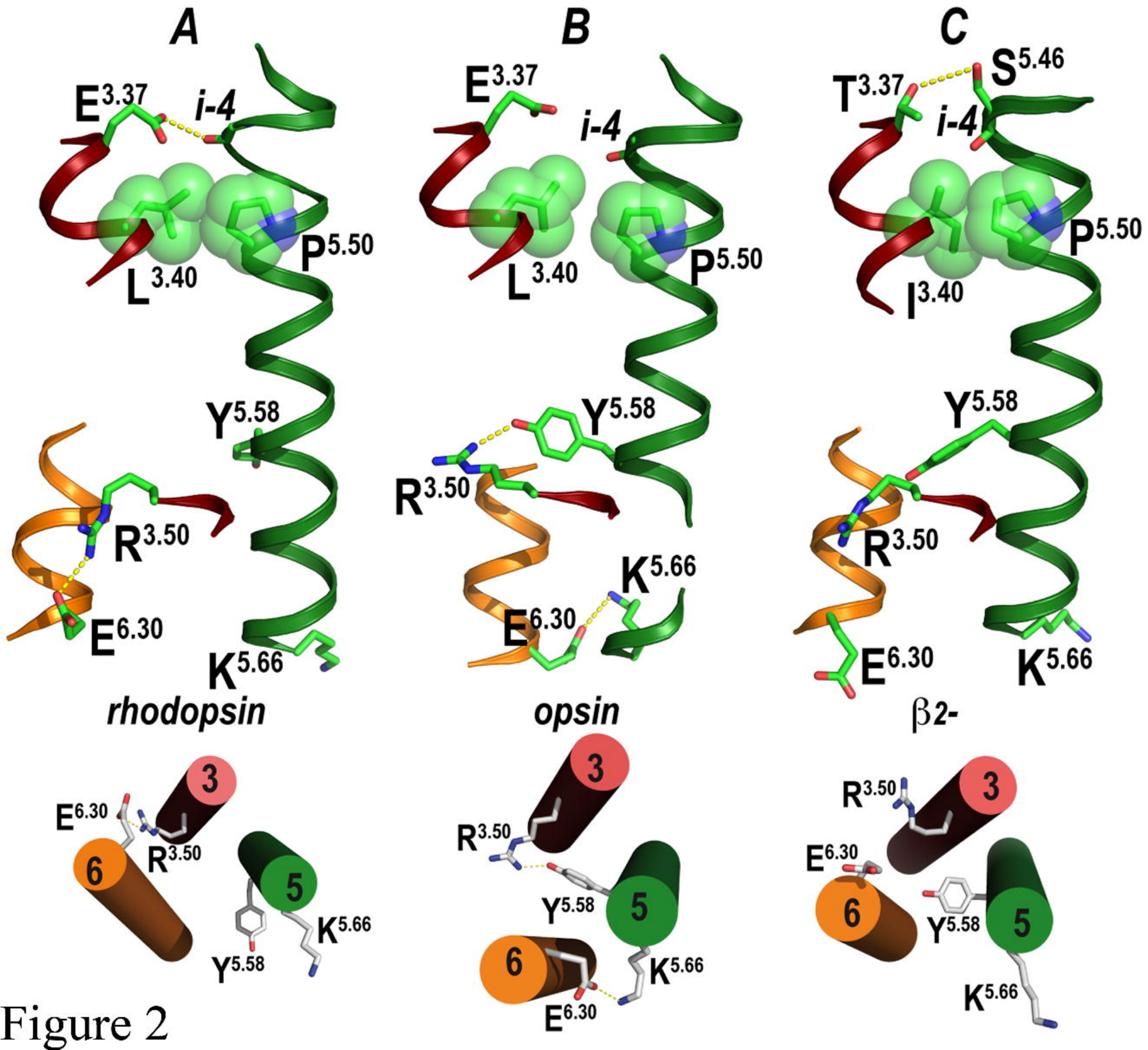
P5.50
β2-

Figure 1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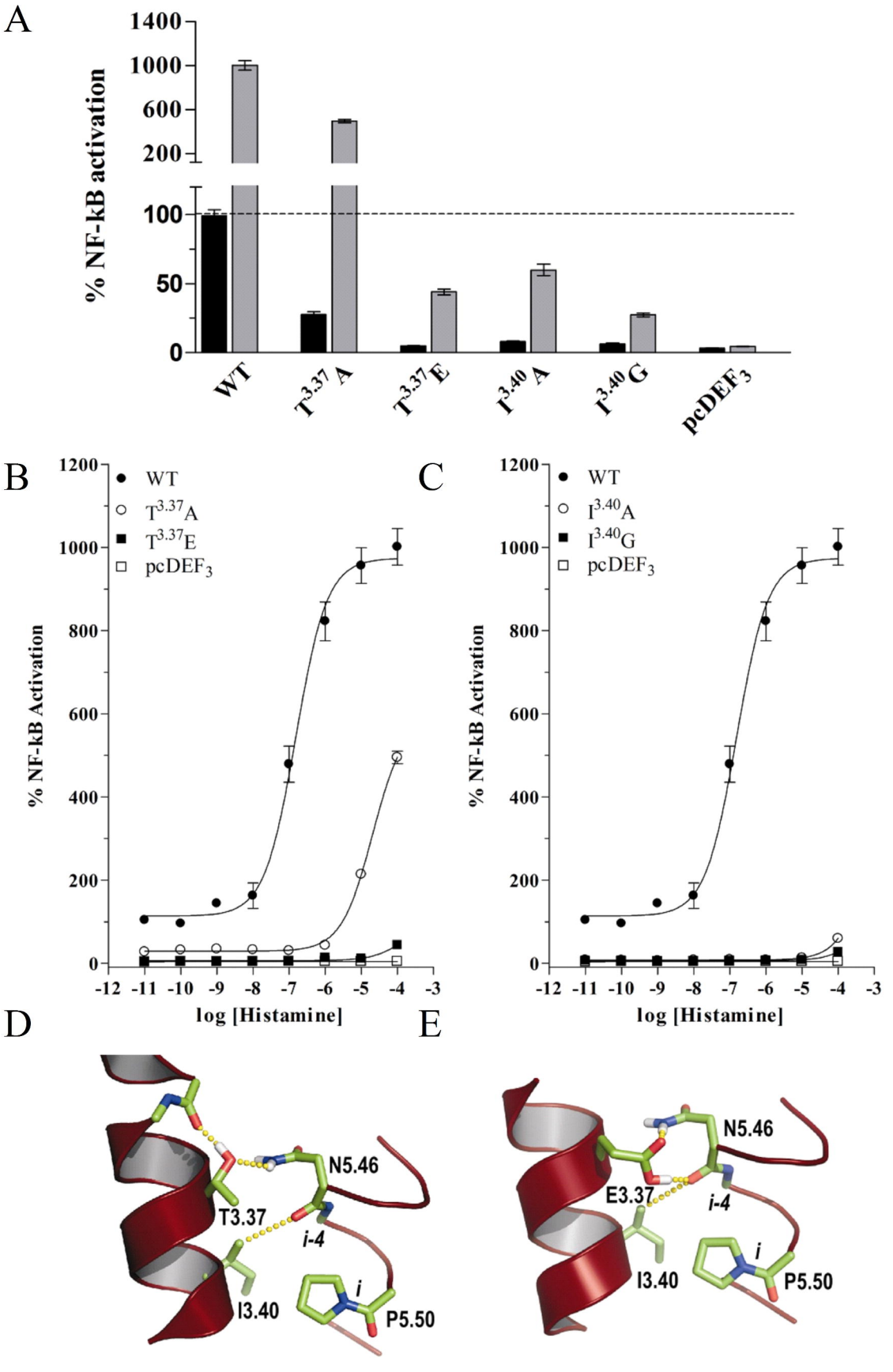
opsin
β2-
3
5
6

Figure 2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1000
600
200
100
50
0
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T3.37 A
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I3.40 A
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pcDEF3
B
WT
T3.37A
T3.37E
pcDEF3
% NF-kB Activation
log [Histamine]
C
WT
I3.40A
I3.40G
pcDEF3
% NF-kB Activation
log [Histamine]
D
N5.46
T3.37
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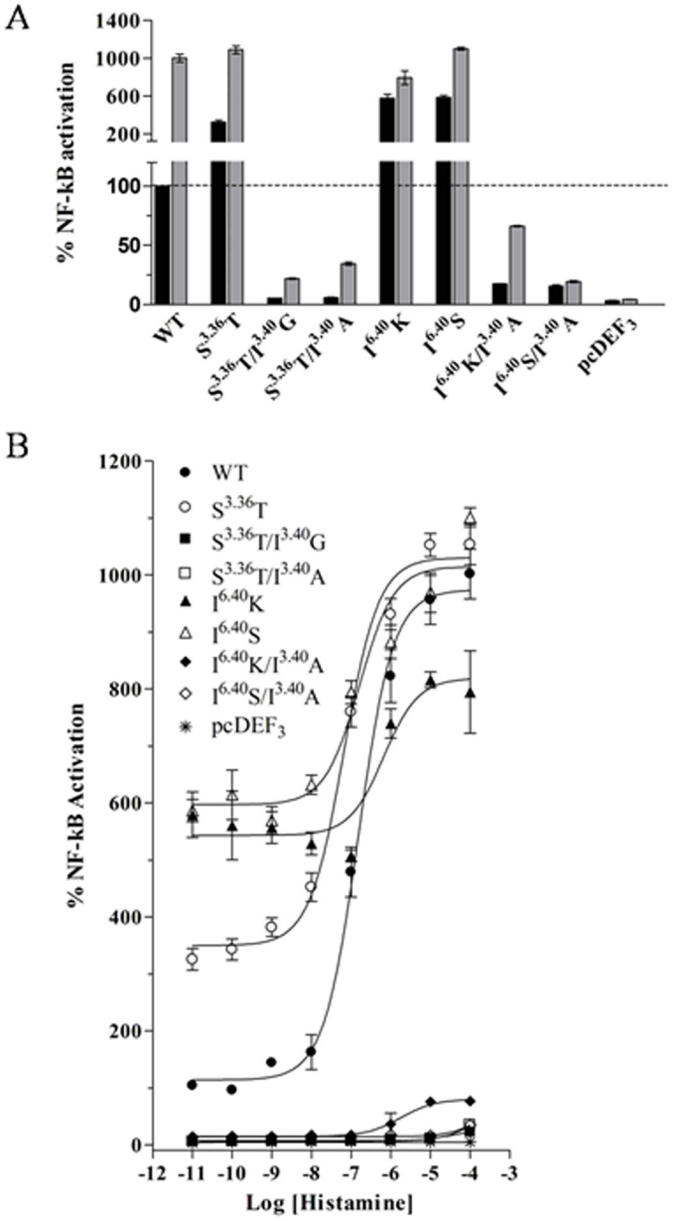
P5.50

Figure 4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1000
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200
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WT
S3.36T
S3.36T/I3.40G
S3.36T/I3.40A
I6.40K
I6.40S
I6.40K/I3.40A
I6.40S/I3.40A
pcDEF3
B
% NF-kB Activation
1200
1000
800
600
400
200
0
WT
S3.36T
S3.36T/I3.40G
S3.36T/I3.40A
I6.40K
I6.40S
I6.40K/I3.40A
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pcDEF3
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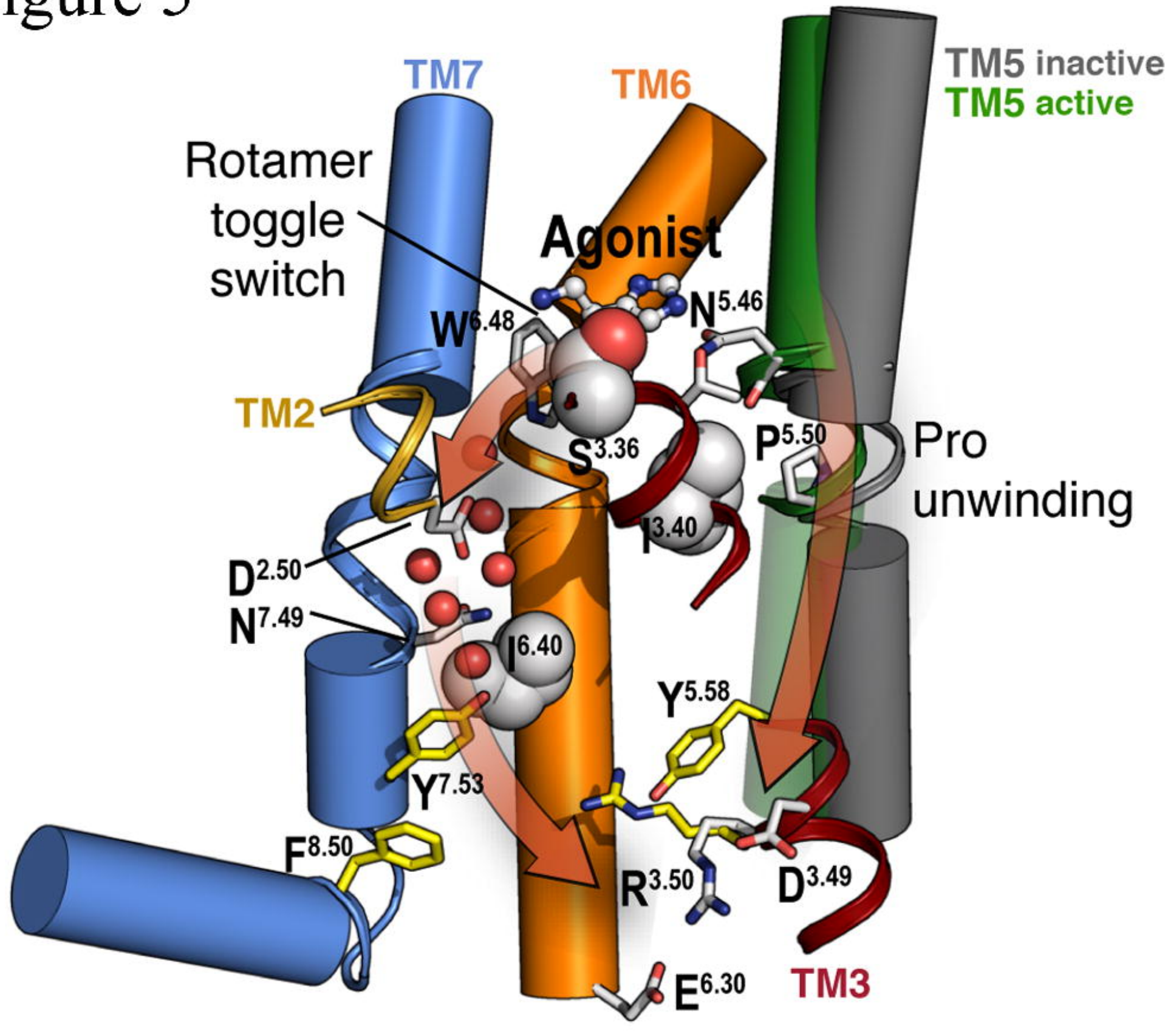
Log [Histamine]

Figure 5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TM6
TM5 inactive
TM5 active
Rotamer toggle switch
Agonist
W6.48
N5.46
TM2
S3.36
P5.50
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D2.50
N7.49
I6.40
Y5.58
Y7.53
F8.50
R3.50
D3.49
E6.30
TM3