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Abstract: The aim of this study was an in-silico analysis of the interaction of the human $\beta 2$ adrenergic receptor with G α s. In a first step, a systematic surface-interaction-scan between the inactive, or active human $\beta 2$ adrenergic receptor and G α s was performed in order to get knowledge about energetically preferred areas on the potential energy surface. Subsequently, two energetically favoured regions for the active human $\beta 2$ adrenergic receptor - G α s - complex were identified. Two representative complex structures were put into a POPC bilayer and solvated in order to perform molecular dynamic simulations. The simulations revealed that both conformations, with comparable potential energy, are stable. A mean number of about 14 hydrogen bonds were observed between the active receptor and G α s for both conformations. Based on these results, it can be suggested that there are two energetically favoured $\beta 2$ -G α s-complexes.

Response to Reviewers: Comments for Reviewer 1

1.1) Construction and surface scan

a) in-house surface scan routine

This routine is very easy and compact. It is described shortly on page 5.

b) Increments used for translation and rotation

The increments are now described on page 5 in detail.

c) Minimization parameters

Parameters, used for minimization are now described in more detail at the beginning of page 6.

d) We understand that it would be useful for some readers to put the pdb coordinates of the starting structure in the online supplementary. On the other hand, this is a highly competitive work, especially with doing the same calculations for other GPCRs, as we plan to do. Thus we do not like to give the starting structure anonymous to every reader.

1.2) Details of molecular dynamics

We now described the dynamics in more detail in the section "Molecular dynamics simulations of the h $\beta 2$ 2RG α scomplex including the natural surrounding" on pages 6 and 7. As the reviewer recommended, we now described the water model, the force field, performance of energy minimization, timestep in dynamics, boundary conditions, integration algorithm, etc.

2) Details of MD simulation protocol

2.1) Berendsen thermostat

As the reviewer has recommended, we repeated the simulations, using the nose-hoover thermostat instead of the berendsen thermostat for temperature coupling and the parrinello-rahman thermostat for pressure coupling in the productive phase. However, a comparison of the simulations with the berendsen and nose-hoover thermostat revealed only small differences with regard to the number of hydrogen bonds and the hydrogen bond network itself between receptor and Galpha subunit. We decided, not to compare the results of the berendsen and nose-hoover thermostat in supplementary, because the intention of this manuscript is not to compare different simulation parameters, but distinct interactions between receptor and Galpha subunit.

2.2) Length of the simulations

It is correct, that the simulation time of the productive phase with 1 ns is short. As reviewer 1 has recommended, we performed a little longer MD. Now have an equilibration phase of 2.55 ns and a productive phase of 2.5 ns. However, the productive phase of 2.5 ns is also very short. But two points have to be taken into account: The simulation box, including receptor, Galpha subunit and surrounding includes about 110000 sites, which leads to extensive computational costs. Additionally, to observe large conformational changes between receptor and Galpha subunit, simulation times of about 1000 ns may be expected. On the other hand the intension of the MD simulations was to refine the hydrogen bond network between receptor and Galpha subunit, including the surrounding, in order to avoid gas phase artefacts. But we are aware, that no large conformational changes between receptor and Galpha could be observed within the simulation time we have used. However, we now have discussed the shortness of the simulation time in more detail in the last paragraph of the section "Hydrogen bond network between h β 2R and G β s." at the end of page 10 and the beginning of page 11.

3) Potential limitations of this study

We now discuss more detailed the potential limitations of this study. Especially in the last paragraph of the section "Hydrogen bond network between h β 2R and G β s." at the end of page 10 and the beginning of page 11.

4) Further citations

We thank the reviewer for recommendation of the additional citations. Now we have introduced the citations into the manuscript.

5) Use of hypens

We deleted the hypen in "in silico", as recommended

6) Explanation of acronyms

We explained acronyms like GDP, GTP and POPC at the time of their first use.

7) Hydrogen bond network: Introduction of a table

We have now removed the description of distinct hydrogen bonds between receptor and Galpha from the section "Hydrogen bond network between h β 2R and Galpha". Instead we have introduced table 1, containing the same information. Additionally, we give detailed information about the frequency of each mentioned hydrogen bond within the productive phase of the simulation.

8) this point is missing in the reviewers comments

9) Length of I3-loop and C-terminus - and their modelling

It is correct, that we did not completely model the I3-loop and the C-terminus of the h β 2R, since there is no adequate information about their conformation available. Thus, a serious modelling is not possible. However, the crystal structure of the h β 2R includes structural information about amino acids in the beginning and end of I3-loop and for some first amino acids of the C-terminus. These amino

acids were included into the modelling and only these amino acids were mentioned with regard to distinct interactions between receptor and Galpha subunit. Now we exactly declared the missing amino acids on page 4. However, we introduced a hypothesis about possible interactions between I3-loop or C-terminus of the receptor with the G protein. This is only a hypothesis, based on geometrical consideration. We have emphasized at the end of page 12 that this is a hypothesis which has to be checked with further experimental or in silico studies.

10) Listing amino acids for example as Thr2.39

This notation was correct. It was the Ballesteros nomenclature. However, we have removed this notation and substituted the numbering with the consecutive numbering given in figure 1. You are right, that the switch between two different numbering schemes may be hard to read.

11) Model I and II are introduced in the text without being explained

We now have explained model I and II at the point of their introduction in the text. In this context, we changed the order of the figures. The previous Fig. 6 is now Fig. 4 and used to explain model I and II graphical. Thus, the figures have changed in the following way: Fig. 4 to Fig. 5, Fig. 5 to Fig. 6 and Fig. 6 to Fig. 4.

12) Last sentence in the abstract

We removed the last sentence "Furthermore, it can be ... one of both models is preferred". It is correct, that this can not be understood in the abstract. A further explanation of this sentence would enlarge the abstract. Anyhow, the hypothesis is given with a detailed description in the manuscript.

13) Several statements in the introduction should be supported by appropriate references.

Now we have supported the three statements in the introduction with appropriate references. This are now references [1], [4, 5] and [6-11].

14) Proof reading for the sake of improved clarity

We have done a careful proofreading to increase clarity. In this context we introduced some lingual changes.

Comments for Reviewer 2

In order to explain "model I" and "model II" with the use of a figure, we changed the previous Fig. 6 to Fig. 4. Thus, the figures have changed in the following way: Fig. 4 to Fig. 5, Fig. 5 to Fig. 6 and Fig. 6 to Fig. 4. Additionally, the methods, like surface scan, minimization parameters and parameters used for MD simulation were described in more detail. Important hydrogen bond interactions between receptor and Galpha subunit are now described in more detail in the new table 1. Reviewer 1 wanted to repeat the calculations with an other thermostat. Now instead of the berendsen thermostat we used the nose-hoover thermostat. Thus, we substituted the hydrogen-bond interactions, calculated with the berendsen thermostat, by the hydrogen-bond interactions, calculated with the nose-hoover thermostat. However, there are only small differences with regard to hydrogen-bond network between receptor and Galpha subunit between the previous and new calculations.

Distinct interactions between the human adrenergic β_2 receptor and $G\alpha_s$ - an *in silico* study

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Abstract

The aim of this study was an *in silico* analysis of the interaction of the human β_2 adrenergic receptor with $G\alpha_s$. In a first step, a systematic surface-interaction-scan between the inactive or active human β_2 adrenergic receptor and $G\alpha_s$ was performed in order to get knowledge about energetically preferred areas on the potential energy surface. Subsequently, two energetically favored regions for the active human β_2 adrenergic receptor – $G\alpha_s$ – complex were identified. Two representative complex structures were put into a POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine) bilayer and solvated in order to perform molecular dynamic simulations. The simulations revealed that both conformations, with comparable potential energy, are stable. A mean number of about 14 hydrogen bonds were observed between the active receptor and $G\alpha_s$ for both conformations. Based on these results, it can be suggested that there are two energetically favored β_2 - $G\alpha_s$ -complexes.

Keywords Adrenergic β_2 receptor • $G\alpha_s$ subunit • Receptor-G-protein interaction • Energy surface scan • Molecular dynamics

Introduction

G protein-coupled receptors (GPCRs) are important targets for several drugs [1]. They consist of seven transmembrane (TM) domains, connected by intra- and extracellular loops and couple intracellular to G proteins, which consist of a $G\alpha$, $G\beta$ and $G\gamma$ subunit [2, 3]. These heterotrimeric G proteins are switches on molecular level, which turn on intracellular signal cascades, induced by activation of the corresponding GPCR [4, 5]. The α subunit ($G\alpha$) is able to switch between its inactive GDP (guanosindiphosphate)-bound conformation and its active GTP (guanosintriphosphate)-bound conformation. Recent crystallographic studies gave insight into the structure of GPCRs on the one hand and G proteins on the other hand [6-11]. But still, there is no crystal structure of a complete GPCR – G protein – complex available. However, some experimental studies give hints to the interactions between GPCR and $G\alpha$ -subunit of the G protein [12].

Two opposite models with regard to GPCR – G protein – coupling are supposed. One model, the “collision coupling” model suggests that G-proteins only interact with receptors in the active state [13]. In contrast, the second model suggests that G proteins are able to interact with the GPCR before the GPCR has been activated by an agonist. This means, GPCR and G protein are pre-coupled. Several studies provide the pre-coupled model [14- 16]. Experimental studies identified the regions of GPCR and G-protein, which interact with each other. It is supposed that during activation of the GPCR a pocket in the intracellular part of the receptor is opened, which interacts with the C-terminus of the $G\alpha$ subunit [12, 17]. Further mutagenesis studies identified amino acids of the $\alpha 4$ - $\beta 6$ loop [18-23] and $\alpha 3$ - $\beta 5$ loop [24] to be involved in interaction with the GPCR. Additionally, it was shown, that the N-terminus of the $G\alpha$ interacts with GPCRs [20, 25-28]. Thus, the experimental studies give information about receptor-G-protein interaction sites. However, all experimentally detected receptor-G-protein interactions cannot be explained by only one model [29]. Thus, it is suggested that receptor dimers may play a role or sequential interactions between receptor and G-protein take place [30, 31].

Until now, only a few *in silico* studies were performed to get insight into possible interactions between receptor and G-protein [32-37]. The aim of this study was an *in silico* simulation of the interaction between the human adrenergic β_2 receptor ($h\beta_2R$) with the $G\alpha_s$ -subunit. First, a systematic scan of the potential energy surface between inactive or active $h\beta_2R$ and $G\alpha_s$ was performed. A subsequent analysis of the surface scan showed that a large number of

conformations of pre-coupled inactive $h\beta_2R$ - $G\alpha_s$ complexes are localized on the same energetic level. In contrast, for the active $h\beta_2R$ - $G\alpha_s$ -complex two conformations are energetically favored. To get detailed information about distinct interactions between $h\beta_2R$ and $G\alpha_s$, an inactive $h\beta_2R$ - $G\alpha_s$ -complex and the two favored active $h\beta_2R$ - $G\alpha_s$ -complexes were embedded in the natural surrounding, including lipid bilayer and water. Subsequently, molecular dynamic simulations were performed.

Methods

Modeling of the inactive and active conformation of $h\beta_2R$

For modeling the inactive conformation of the $h\beta_2R$ (Fig. 1), we used the corresponding crystal structure (2RH1.pdb) [7-9]. Based on the crystal structure of the active state of opsin, coupled to 11 amino acids of the C-terminus of $G\alpha$ (3DQB.pdb) [12], a model of the active state $h\beta_2R$ was generated by homology modeling. This homology modeling was performed using the software SYBYL 7.0 (Tripos Inc.). The alignment between $h\beta_2R$ and opsin is based on the highly conserved amino acids of each TM domain as described [38]. The loops, showing different length between the $h\beta_2R$ and opsin were modeled using the loop search module of SYBYL 7.0. Since the E2 loop of the $h\beta_2R$ is significantly different to the E2 loop conformation of opsin, as exhibited by the corresponding crystal structures of the inactive $h\beta_2R$ and opsin, the E2 loop was adopted from 2RH1.pdb. Since the crystal structure exhibits no information about the conformation of I3 loop or C-terminus, both were not fully included into the model. We did not model amino acids 232 – 262 of the I3-loop (Fig. 1), instead we closed the I3-loop at by connecting amino acid 231 and 263 by six alanines. The C-terminus was modeled up to amino acid 346 (Fig. 1).

Construction of the $G\alpha_s$ in the GDP-bound state

Since no crystal structure of the GDP-bound state of $G\alpha_s$ is available, a model of the GDP-bound state of $G\alpha_s$ was constructed by comparative homology modeling, using the heterotrimeric GDP-bound state of $G\alpha_{i1}$ (1GP2.pdb) [39] as template. The modeling was performed, as already described [32].

Construction of the $G\alpha_s$ in the GTP-bound state

The crystal structure of $G\alpha_s$ (1AZT.pdb) [40] with GTP γ S bound was used to model the $G\alpha_s$ (Fig. 1) in the GTP bound state. Since there is no complete N-terminus in 1AZT.pdb, 1AZT.pdb was aligned to 1GG2.pdb [41] and the N-terminus of 1GG2.pdb was transferred to 1AZT.pdb. Additionally, the missing amino acids in the C-terminus were added in a helical conformation. Furthermore, the missing amino acids 91-112 were adopted from 1GG2.pdb. Amino acids were mutated into the corresponding amino acid of h $G\alpha_s$, where appropriate.

Construction and surface scan of the inactive and active h β_2 R- $G\alpha_s$ -complexes

An in-house surface-scan routine was used to dock the $G\alpha_s$ to the h β_2 R. Therefore, a starting structure was needed. This was generated by manual positioning of the $G\alpha_s$ below the intracellular part of the inactive or active h β_2 R. In the next step, a surface-scan of the contact surface between the intracellular part of the h β_2 R and the $G\alpha_s$ was performed with the in-house surface-scan routine: Therefore, the position of the h β_2 R was fixed and only the $G\alpha_s$ was translated in x-, y- and z-direction and rotated about the x-, y- and z axis systematically, always using the described starting structure as starting point (Fig. 2). The surface scan was performed for the inactive and the active h β_2 R- $G\alpha_s$ -complex. 9 points with an increment of 0.25 nm were used to scan in x- and y-direction, respectively. In z-direction, 17 points with an increment of 0.125 nm were established. For rotation about z-axis, 12 points with an angle increment of 30 degrees, for rotation about x- and y-axis, 5 points with an increment of 15 degrees were used. Thus, within this scan, about 400000 structures were generated. After analyzing these results with regard to potential energy of the active h β_2 R- $G\alpha_s$ -complex, two distinct regions giving hint to local minima were identified. In order to localize the two minima more exactly, a refined scan was performed in these two regions. Therefore, 9 points with an increment of 0.05 nm were used in x-, y- and z-direction, respectively. The rotation about z-axis was refined by 7 points with an increment of 10 degrees. The rotation about x- and y-axis was refined by 5 points with an increment of 7.5 degrees. Thus, for each of both refined scans, about 125000 structures were calculated. Summarizing up, more than 650000 structures for the active h β_2 R- $G\alpha_s$ -complex were calculated. The surface scan did not reveal significant minima for the inactive h β_2 R- $G\alpha_s$ -complex, thus, a refined scan was not performed. After each structure generation, the resulting h β_2 R- $G\alpha_s$ -complex was completely

energy minimized with Gromacs [42], using the ffG53A6 force field [43] for the protein. Energy minimization, using the steepest decent method, was performed for maximum 10000 steps, or until a maximum force smaller than $100 \text{ kJ mol}^{-1} \text{ nm}^{-1}$ was achieved. The distances for coulomb cutoff and Lennard-Jones cutoff were set to 1.4 nm. Thereby, all sites were allowed to be minimized, this means, no position constraints were set.

Molecular dynamics simulations of the $\text{h}\beta_2\text{R-G}\alpha_s$ -complex including the natural surrounding

To get detailed insight into the interactions between the inactive $\text{h}\beta_2\text{R}$ and the GDP-bound state of $\text{G}\alpha_s$, the complex with lowest potential energy was embedded in a POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine) bilayer (10 nm x 10 nm) containing 218 POPC molecules. Subsequently, the whole simulation box (10 nm x 10 nm x 12 nm) was filled with 30555 spc (single point charge) water [44] molecules. The resulting system, containing about 110000 sites, was energetically minimized (maximum 10000 steps, or until a maximum force smaller than $100 \text{ kJ mol}^{-1} \text{ nm}^{-1}$ was achieved; the distances for coulomb cutoff and Lennard-Jones cutoff were set to 1.4 nm; all sites were allowed to be minimized; no position constraints were set) and then used as a starting structure for the molecular dynamics simulation. The same steps were performed for the two energetically favored active $\text{h}\beta_2\text{R-G}\alpha_s$ -complexes with completely different conformations. All molecular dynamic simulations were performed with Gromacs [42]. For all simulations, rectangular periodic boundary conditions were used. The particle mesh Ewald (PME) method [45] was applied to describe the electrostatic interactions. The distances for coulomb cutoff and Lennard-Jones cutoff were set to 1.4 nm. The dielectric constant was set to 1. For all simulations, a timestep of 2 fs was used. The list of non bonded atoms pair list was updated every 10 fs. The simulation temperature was 310 K and the pressure 1 bar. In the equilibration phase, berendsen temperature (time constant 0.1 ps) and pressure (time constant 0.5 ps) coupling were used [46]. In the subsequent productive phase, the Nose-Hoover thermostat [47, 48] was used for temperature (time constant 1ps) and the Parinello-Rahman barostat (time constant 1 ps) [49] for pressure coupling. A 2550 ps equilibration phase with position constraints onto backbone (bb) and sidechain (sc) atoms of the $\text{h}\beta_2\text{R}$ and $\text{G}\alpha_s$ was performed. Therefore, the simulation was divided into 12 cycles with the following position constraints in $\text{kJ}/(\text{mol nm}^2)$: cycle 1: 500 ps, bb 5000, sc 5000; cycle 2: 250 ps, bb 5000, sc 4000; cycle 3: 250 ps, bb 4000, sc 3000; cycle 4: 250 ps, bb 3000, sc 2000; cycle 5: 150 ps, bb 2000, sc 1000; cycle 6: 150 ps,

bb 1000, sc 800; cycle 7: 150 ps, bb 800, sc 600; cycle 8: 150 ps, bb 600, sc 400; cycle 9: 150 ps, bb 400, sc 200; cycle 10: 150 ps, bb 200, sc 100; cycle 11: 150 ps, bb 100, sc 0; cycle 12: 250 ps, bb 0, sc 0. After the 2550 ps equilibration phase, a 2500 ps productive phase without any position constraints was performed.

Results and discussion

Surface scan of the inactive and active $h\beta_2R$ - $G\alpha_s$ -complex

The results of the potential energy surface scan are presented in Fig. 3. The section of the energy surface with variation of the distance z between inactive $h\beta_2R$ and $G\alpha_s$ and additionally rotation of $G\alpha_s$ around the z axis (Fig. 3A) shows a wide plain with rather small potential energy for a distance $z > 0.6$ nm of the $G\alpha_s$ C-terminus with respect to the reference position. An increasing distance z leads to a decrease in potential energy. In contrast, for a decrease of z , a significant increase in potential energy was observed. This increase in potential energy can be explained by the fact that there is not enough space between the intracellular parts of the TM domains of the inactive $h\beta_2R$. Consequently, the C-terminus of $G\alpha_s$ cannot penetrate between the helices of the receptor. For $z = 0.075$ nm, the potential energy as function of translation of the $G\alpha_s$ in x - and y -direction is given (Fig. 3B). Here, the potential energy is a wide plain, with a significant increase at only some positions. This result can be explained by the fact, that the $G\alpha_s$ shows only a slight contact to the inactive $h\beta_2R$, without penetration of the C-terminus between the TM domains at the given z . Thus, the $G\alpha_s$ can move on the intracellular receptor surface without steric collisions. The potential energy surface of the active $h\beta_2R$ and $G\alpha_s$, with a variation of the distance z between receptor and $G\alpha_s$ and a rotation of $G\alpha_s$ around the z axis was significantly different to that of the inactive $h\beta_2R$ (Fig. 3C). Minima in potential energy were found for $z < 0.5$ nm, especially for values of z between 0.0 nm and 0.25 nm. For distances z in this range, only a slight rotation of the $G\alpha_s$ around the z axis, without large increase in potential energy was possible. This minimum is defined as model I (Fig. 4). Therein, the N-terminus of the $G\alpha_s$ is located below TM II of the receptor. The $G\alpha_s$ subunit is located in the large space between TM VI and TM VII of the receptor (Fig. 4). An increase of the distance $z > 0.6$ nm led to a slight increase in potential energy, due to the fact, that for a larger distance the interaction energy between the active

$h\beta_2R$ and the $G\alpha_s$ was lost. For z from about 0.6 nm to 1.0 nm, a second local, but energetical higher minimum area was identified for γ ranged between $210^\circ - 240^\circ$. In this small area further $h\beta_2R$ - $G\alpha_s$ -complex can be located, but due to the z from 0.6 nm to 1.0 nm, there was no penetration of the $G\alpha_s$ -C-terminus between the TMs of the receptor, as indicated by the crystal structure. But further investigations at γ , ranged between $210^\circ - 240^\circ$, but different x , y , α and β , identified another local minimum, with possible penetration depth of the C-terminus of $G\alpha_s$ around $z \approx 0$ (corresponding section of the energy surface not shown). This minimum is defined as model II (Fig. 4). Therein, the N-terminus of the $G\alpha_s$ is located between TM VI and TM VII of the receptor and the $G\alpha_s$ subunit is located below TM V and TM VI of the receptor (Fig. 4). In general, by the potential surface scan, two energetically favored minima on the potential energy surface were found. This corresponds to two different binding models for the active $h\beta_2R$ - $G\alpha_s$ -complex. For $z = 0.25$ nm the potential energy with translation of $G\alpha_s$ in x - and y -direction is shown (Fig. 3D). In contrast to the inactive complex, the potential energy increased significantly with small translation steps in x - or y -direction. This is in good accordance to the fact, that a part of the $G\alpha_s$ C-terminus is located in a small pocket between the TM domains of the receptor. Thus, small translations led to collisions with the receptor. The regions, dependent of distance z and rotation about z axis, which are more stable for the inactive $h\beta_2R$ - $G\alpha_s$ - (blue) and active $h\beta_2R$ - $G\alpha_s$ -complex (red), respectively are given (Fig. 3E). The potential energy for $y = 0$ nm with variation of z is given (Fig. 3F). The inactive $h\beta_2R$ - $G\alpha_s$ -complex has, compared to the active $h\beta_2R$ - $G\alpha_s$ -complexes (model I and II), lower potential energy for large values of $z > 0.85$ nm. But for values of $z < 0.75$ nm the potential energy of the active $h\beta_2R$ - $G\alpha_s$ -complex is smaller, compared to the inactive $h\beta_2R$ - $G\alpha_s$ -complex.

Hydrogen bond network between $h\beta_2R$ and $G\alpha_s$

To get detailed insight into the hydrogen bond networking (Fig. 5) between the inactive $h\beta_2R$ and $G\alpha_s$ on the one hand and the active $h\beta_2R$ and $G\alpha_s$ on the other hand, molecular dynamics simulations were performed. For the inactive $h\beta_2R$ - $G\alpha_s$ -complex only a small number of hydrogen bonds, ranged between 0 to 7 were observed. This corresponds to a mean number of about 3 hydrogen bonds (Fig. 5A). In contrast, for the active $h\beta_2R$ - $G\alpha_s$ -complex, according to model I, during the 2.5 ns productive phase of the MD simulation a mean number of about 12 hydrogen bonds were detected (Fig. 5B). In general, these hydrogen bonds were established

by 38 different amino acid interactions between receptor and $G\alpha_s$ (Fig. 5C). Additionally, the frequency of a hydrogen bond during the productive phase is given in Table 1. As indicated (Table 1, Fig. 5C), for model I, only a number of about 11 distinct hydrogen bond interactions between receptor and $G\alpha_s$ is observed during more than 50 % and a number of about 6 during more than 80 % of the simulation. In Fig. 6, the hydrogen bond interactions between $h\beta_2R$ and $G\alpha_s$ with a frequency > 50 % are presented for model I. For the active $h\beta_2R$ - $G\alpha_s$ -complex, according to model II, a mean number of about 15 hydrogen bonds were detected during the 2.5 ns productive phase of the simulation (Fig. 5D). These hydrogen bonds were established by 38 different amino acid interactions between receptor and $G\alpha_s$ (Fig. 5E). However, only a number of about 13 distinct hydrogen bond interactions between receptor and $G\alpha_s$ is observed during more than 50 % and a number of about 11 during more than 80 % of the simulation (Table 1, Fig. 5E). In Fig. 6, the hydrogen bond interactions between $h\beta_2R$ and $G\alpha_s$ with a frequency > 50 % are presented for model II.

In general, it should be taken into account that the productive phases of the MD simulations with 2.5 ns are too short to observe large conformational changes between receptor and $G\alpha_s$. One reason for this short simulation time is the large size of the simulation box and computational capacity on the one hand. On the other hand, large conformational changes between receptor and G-protein may be expected for simulation times longer than 1000 ns. Nowadays, such high cost simulations are nearly not realizable because of the large computation time. However, the MD simulations in this study were performed in order to get information about a hydrogen bond network between receptor and $G\alpha_s$, taking into account the natural surrounding, like water and lipid bilayer, of the receptor- $G\alpha_s$ complex. The surrounding is necessary to avoid gas phase artifacts with regard to the hydrogen bond network in the receptor- $G\alpha_s$ -complex. However, one should be aware that both interaction models are primary based on gas phase scans of potential energy surface and were refined by MD simulations including the natural surrounding. On the other hand, it is important to get more detailed insights into the interactions between GPCR and G protein on molecular level. Thus, both models, presented here may represent a good starting point for further studies.

Two models for receptor-G-protein interaction

Based on the calculations it can be suggested that two different $h\beta_2R$ - $G\alpha_s$ -complexes are energetically favored (Fig. 4). In model I (Fig. 4), the N-terminus of the $G\alpha_s$ is located below

TM II of the receptor. Thus, the front of the $G\alpha_s$ subunit, interacting with $G\beta\gamma$, is located in the large space between TM VI and TM VII of the receptor. Therefore, it can be suggested that a long C-terminus of the receptor may be able to interact with the $G\beta\gamma$ subunit, subsequently leading to a stabilization of the receptor-G-protein-complex (Fig. 4). In contrast, in model II (Fig. 4), the N-terminus of the $G\alpha_s$ is located between TM VI and TM VII of the receptor. Consequently, the front of the $G\alpha_s$ subunit, interacting with $G\beta\gamma$, is located below TM V and TM VI of the receptor. Based on this data it can be suggested, that a long I3 loop of the receptor can interact with the $G\beta\gamma$ subunit, again leading to a stabilization of the receptor G-protein-complex (Fig. 4). Additionally, an analysis of the number of amino acids in the I3-loop or C-terminus of the human aminergic GPCRs was performed (Fig. 7). This analysis revealed that the I3 of the $G\alpha_s$ -coupling human aminergic GPCRs is significantly shorter, than the C-terminus. In contrast, the I3 of the $G\alpha_i$ -coupling human aminergic GPCRs is significantly longer, than the C-terminus, whereas no significant difference in length of I3 and C-terminus was found for the $G\alpha_q$ -coupling GPCRs. These data may indicate that the preferred orientation (model I or II, Fig. 4) between receptor and $G\alpha$ -subunit is dependent from length of I3-loop or C-terminus. Furthermore, it may be speculated, that the $G\alpha_s$ couple, because of the longer C-terminus according to model I, whereas the $G\alpha_i$ couple, because of the longer I3-loop according to model II to the corresponding receptor.

Comparison to experimental results

In several experimental and theoretical studies, the interactions between biogenic amine receptor and $G\alpha$ subunit have been analyzed. However, there are only some hints to distinct interactions between receptor and $G\alpha$ subunit. Several experimental studies suggest that the C-terminus [12, 17], $\alpha 4$ - $\beta 6$ loop [18-23], $\alpha 3$ - $\beta 5$ loop [24] and also the N-terminus [20, 25-28] of $G\alpha$ subunits are relevant for receptor- $G\alpha$ -interaction. Oldham and Hamm [29] point out that experimental results give information about a putative receptor- $G\alpha$ -binding-surface. However, all experimental results cannot be explained with only one model. Thus, one hypothesis is that these identified regions interact not at one time, but sequentially with the receptor [30]. Instead, based on our results we present an alternative hypothesis: There are two possible interaction models (model I and model II, Fig. 4), but which model is established, is suggested to be dependent on length of I3-loop or C-terminus. It can be hypothesized that receptors with long C-terminus interact with the G-proteins according to

model I (Fig. 4) and receptors with long I3-loop with the G-proteins according to model II (Fig. 4). It should be emphasized that this is only a hypothesis, since the conformation of long I3 loops or C-termini cannot be modeled due to the lack of an adequate crystal structure. However, the mentioned hypothesis is based on geometrical considerations with regard to orientation of receptor and $G\alpha$ subunit to each other. Further experimental and *in silico* studies have to be performed, to check this hypothesis. Additionally it has to be taken into account, that the knowledge about receptor-G-protein interaction is based on a large number of different receptors, interacting with different $G\alpha$ -subunits. To verify our new, alternative hypothesis, extensive and systematic mutagenesis studies have to be carried out with at least three receptor-G-protein systems, coupling to $G\alpha_i$, $G\alpha_q$ and $G\alpha_s$. The adrenergic receptors would be very suitable, since the α_1 adrenergic receptors interact with $G\alpha_q$, the α_2 adrenergic receptors with $G\alpha_i$ and the β adrenergic receptors with $G\alpha_s$. A comparison of our simulation results with experimental data shows in general good accordance. The alignment of the activated βh_2R in complex with the first 11 amino acids of $G\alpha_s$ with the corresponding crystal structure of opsin (3DQB.pdb) revealed an rmsd deviation of about 0.45 nm for the backbone atoms for model I and 1.01 nm for model II. Taking into account, that our most favored structures are based on an energy-surface scan, the deviation for model I is rather small. In model II, the 11 amino acids of the C-terminus have a different orientation to the corresponding amino acids of 3DQB.pdb. Experimental studies have shown that the β_1 -adrenergic receptor preferentially interacts with specific isoforms of $G\beta$ subunit [50]. Thus, both models, suggesting a distinct interaction between C-terminus (model I, Fig. 4) of I3-loop (model II, Fig. 4) of the receptor with the $G\beta$ subunit are in good accordance to these experimental results. Furthermore, experimental data suggest that even the I1-loop and C-terminus interact with G-proteins [51]. In both interaction models described here, an interaction between I1-loop or C-terminus of the receptor with the G-protein was found. This *in silico* result is in good accordance to the experimental results. Another experimental study showed that a peptide, corresponding to the I3-loop of the α_2 -adrenergic receptor can be crosslinked the 60 C-terminal amino acids of the $G\beta$ subunit [26, 52]. This experimental result is on the one hand in good accordance to model II (Fig. 4). On the other hand it agrees with our hypothesis that the orientation between receptor and G-protein is dependent on the length of I3-loop or C-terminus. Based on the hypothesis, introduced here, the α_2 -adrenergic receptor, possessing a long I3-loop, is suggested to interact with the G-protein according to model II (Fig. 4). Consequently, an interaction between I3-loop and $G\beta$ subunit is expected.

Summarizing up, it can be concluded that both receptor – G-protein interaction models, presented here, are in good accordance to experimental data. Although, it has to be taken into account that both models base on a gas phase scan of potential energy between receptor and $G\alpha$ subunit, both final *in silico* models for the $h\beta_2R$ - $G\alpha_s$ -complexes, taking into account the lipid bilayer and solvent, may represent a good starting point for future theoretical and experimental studies.

Conclusions

Based on the crystal structure of activated opsin, in complex with 11 amino acids of the G_t -C-terminus, two models of the active $h\beta_2R$, in complex with $G\alpha_s$ in the GTP-bound state were generated. The models based on gas phase energy-surface scans and were refined by molecular dynamics simulations, including the natural surrounding, like POPC bilayer and water. In general, the two favored active $h\beta_2R$ - $G\alpha_s$ models are in good accordance to several experimental data, described in literature. Thus, both models may give detailed insight into distinct interactions between $h\beta_2R$ and $G\alpha_s$. Besides that, it may be hypothesized that there exist two different interaction states between receptor and G-protein, in dependence of length of I3-loop or C-terminus. Furthermore it gives very useful hints, with regard to future mutagenesis studies, for further experimental verification.

References

1. Wise A, Gearing K, Rees S (2002) Target validation of G-protein coupled receptors. *Drug Discovery Today* 7:235-246
2. 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
3. Oldham WM, Hamm HE (2006) Structural basis of function in heterotrimeric G proteins. *Q Rev Biophys* 39:117-166
4. Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol.* 3:639-650
5. Gether U, Kobilka BK (1998) G Protein-coupled receptors II. Mechanism of agonist activation 273:17979-17982
6. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okado T, Stenkap RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289:739-745
7. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human β_2 -adrenergic G protein-coupled receptor. *Science* 318:1258-1265
8. Rasmussen SGF, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VRP, Sanishvili R, Fischetti RF, Schertler GFX, Weis WI, Kobilka BK (2007) Crystal structure of the human β_2 adrenergic G-protein-coupled receptor. *Nature* 450:383-387
9. Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into β_2 -adrenergic receptor function. *Science* 318:1266-1273
10. Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, Schertler GF (2008) Structure of a β_1 -adrenergic G protein-coupled receptor. *Nature* 454:486-491
11. Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien YET, Lane JR, Ijzerman AP, Stevens RC (2008) The 2.6 Å crystal structure of a human A_{2A} adenosine receptor bound to an antagonist. *Science* 322:1211-1217

12. Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauß N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 455:497-503
13. Tolkovsky AM, Levitzki A (1978) Mode of coupling between the β -adrenergic receptor and adenylate cyclase in turkey erythrocytes. *Biochemistry* 17:3795-3810
14. Gales C, van Durm JJJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G-protein complexes. *Nat Struct Mol Biol* 13:778-786
15. Alves ID, Salamon Z, Varga E, Yamamura HI, Tollin G, Hruby VJ (2003) Direct observation of G-protein binding to the human δ -opioid receptor using plasmon-waveguide resonance spectroscopy. *J Biol Chem* 278:48890-48897
16. Alves ID, Salgado GFJ, Salamon Z, Brown MF, Tollin G, Hruby VJ (2005) Phosphatidylethanolamine enhances rhodopsin photoactivation and transducin binding in a solid supported lipid bilayer as determined using plasmon-waveguide resonance spectroscopy. *Biophys J* 88:198-210
17. Janz JM, Farrens DL (2004) Rhodopsin activation exposes a key hydrophobic binding site for the transducin α -subunit C terminus. *J Biol Chem*. 279:29767-29773
18. Lichtarge O, Bourne HR, Cohen FE (1996) Evolutionarily conserved $G\alpha\beta\gamma$ binding surfaces support a model of the G protein-receptor complex. *Proc Natl Acad Sci USA* 93:7507-7511
19. Mazzoni MR, Hamm HE (1996) Interaction of transducin with light-activated rhodopsin protects it from proteolytic digestion by trypsin. *J Biol Chem*. 271:30034-30040
20. Onrust R, Herzmark P, Chi P, Garcia PD, Lichtarge O, Kingsley C, Bourne HR (1997) Receptor and $\beta\gamma$ binding sites in the α subunit of the retinal G protein transducin. *Science* 275:381-384
21. Bae H, Anderson K, Flood LA, Skiba NP, Hamm HE, Graber SG (1997) Molecular determinants of selectivity in 5-hydroxytryptamine_{1B} receptor-G protein interactions. *J Biol Chem* 272:32071-32077
22. Bae H, Cabrera-Vera TM, Depree KM, Graber SG, Hamm HE (1999) Two amino acids within the $\alpha 4$ helix of G_{i1} mediate coupling with 5-hydroxytryptamine_{1B} receptors. *J Biol Chem* 274:14963-14971
23. Cai K, Itoh Y, Khorana HG (2001) Mapping of contact sites in complex formation between transducin and light-activated rhodopsin by covalent crosslinking: use of a photoactivatable reagent. *Proc Natl Acad Sci USA* 98:4877-4882

24. Grishina G, Bertlot CH (2000) A surface-exposed region of G_{sα} in which substitutions decrease receptor-mediated activation and increase receptor affinity. *Mol Pharmacol* 57:1081-1092
25. Hamm HE, Deretic D, Arendt A, Hargrave PA, Koenig B, Hofmann KP (1988) Site of G protein binding to rhodopsin mapped with synthetic peptides from the α subunit. *Science* 241:832-835
26. Taylor JM, Jacob-Mosier GG, Lawton RG, Remmers AE, Neubig RR (1994) Binding of an α₂ adrenergic receptor third intracellular loop peptide to Gβ and the amino terminus of Gα. *J Biol Chem* 269:27618-27624
27. Ho MK, Wong YH (2000) The amino terminus of Gα_z is required for receptor recognition, whereas its α4/β6 loop is essential for inhibition of adenylyl cyclase. *Mol Pharmacol* 58:993-1000
28. Itoh Y, Cai K, Khorana HG (2001) Mapping of contact sites in complex formation between light-activated rhodopsin and transducin by covalent crosslinking: use of a chemically preactivated reagent. *Proc Natl Acad Sci USA* 98:4883-4887
29. Oldham WM, Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9:60-71.
30. Herrmann R, Heck M, Henklein P, Henklein P, Kleuss C, Hofmann KP, Ernst OP (2004) Sequence of interactions in receptor-G protein coupling. *J Biol Chem.* 279:24283-24290.
31. Herrmann R, Heck M, Henklein P, Hofmann KP, Ernst OP (2006) Signal transfer from GPCRs to G proteins: role of the Gα N-terminal region in rhodopsin-transducin coupling. *J Biol Chem* 281:30234-30241
32. Fanelli F, Menziani C, Scheer A, Cotecchia S, de Benedetti PG (1999) Theoretical study of the electrostatically driven step of receptor-G protein recognition. *PROTEINS: Structure, Function and Genetics* 37:145-156
33. Fanelli F, Menziani C, Scheer A, Cotecchia S, de Benedetti PG (1999) Theoretical study on receptor-G protein recognition: New insights into the mechanism of the α_{1b}-adrenergic receptor activation. *Int J Quant Chem* 73:71-83
34. Greasley PJ, Fanelli F, Scheer A, Abuin L, Nenniger-Tosato M, de Benedetti PG, Cotecchia S (2001) Mutational and computational analysis of the α_{1B}-adrenergic receptor: involvement of basic and hydrophobic residues in receptor activation and G protein coupling. *J Biol Chem* 276:46485-46494

35. Oliveira L, Paiva PB, Paiva ACM, Vriend G (2003) Sequence analysis reveals how G protein-coupled receptors transduce the signal to the G protein. *PROTEINS: Structure, Function and Genetics* 52:553-560
36. Chou KC (2005) Coupling interaction between thromboxane A2 receptor and alpha-13 subunit of guanine nucleotide-binding protein. *J Proteome Res* 4:1681-1686
37. Raimondi F, Seeber M, de Benedetti PG, Fanelli F (2008) Mechanisms of the inter- and intramolecular communication in GPCRs and G proteins. *J Am Chem Soc* 130:4310-4325
38. Ballesteros JA, Shi L, 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
39. Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR (1995) The structure of the G protein heterotrimer $G\alpha_{i1}\beta_1\gamma_2$. *Cell* 83:1047-1058
40. Sunahara RK, Tesmer JJ, Gilman AG, Sprang SR (1997) Crystal structure of the adenylyl cyclase activator $Gs\alpha$. *Science* 278:1943-1947
41. Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR (1995) The structure of the G protein heterotrimer G_i α β 1 γ 2. *Cell* 83:1047-1058
42. Van der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC (2005) GROMACS: fast, flexible, and free. *J Comput Chem* 26:1701-1718
43. Oostenbrink C, Villa A, Mark AE, van Gunsteren WF (2004) A biomolecular force field based on the free enthalpy of hydration and solvation: the GROMOS force-field parameter sets 53A5 and 53A6. *J Comput Chem* 25:1656-1676
44. Berendsen HJC, Postma JPM, van Gunsteren WF, Hermans J (1981) In: Pullmann BE (ed) *Intermolecular Forces*. Reidel: Dordrecht, The Netherlands, p331
45. Essman U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG (1995) A smooth particle mesh Ewald method. *J Chem Phys* 103:8577-8593
46. Berendsen HJC, Postma JPM, van Gunsteren WF, DiNola A, Haak JR (1984) Molecular dynamics with coupling to an external bath. *J Chem Phys* 81:3684-3690
47. Nose S (1984) A molecular-dynamics method for simulations in the canonical ensemble. *Mol Phys* 52:255-278
48. Hoover WG (1985) Canonical dynamics – equilibrium phase-space distributions. *Phys. Rev A* 31:1695-1697

49. Parrinello M, Rahman A (1981) Polymorphic transitions in single crystals – a new molecular-dynamics method. *J Appl Phys* 52:7182-7190
50. McIntire WE, MacCleery G, Garrison JC (2001) The G protein β subunit is a determinant in the coupling of G_s to the β_1 -adrenergic and A_{2a} adenosine receptors. *J Biol Chem* 276:15801-15809
51. Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J* 11:346-354
52. Taylor J M, Jacob-Mosier GG, Lawton RG, van Dort M, Neubig RR (1996) Receptor and membrane interaction sites on $G\beta$. A receptor-derived peptide binds to the carboxyl terminus. *J Biol Chem* 271:3336-3339

Tables

Table 1 Hydrogen bond interactions between h β_2 R and G α_s

	model I		model II	
	interaction sites h β_2 R - G α_s	[%]	interaction sites h β_2 R - G α_s	[%]
1	63R (I1) – 264N ($\alpha 2$ - $\beta 4$)	0.2	63R (I1) – 409Q ($\alpha 5$)	10.3
2	63R (I1) – 265D ($\alpha 2$ - $\beta 4$)	83.9	63R (I1) – 412H ($\alpha 5$)	82.3
3	63R (I1) – 415Q (C-term)	0.2	63R (I1) – 66E (αN - $\beta 1$)	51.5
4	66T (I1) – 417E (C-term)	0.1	66T (I1) – 414R (C-term)	0.2
5	68T (TMII) – 416Y (C-term)	0.2	68T (TMII) – 414R (C-term)	0.2
6	68T (TMII) – 417E (C-term)	99.2	68T (TMII) – 418L (C-term)	0.2
7	69N (TMII) – 417E (C-term)	86.1	69N (TMII) – 417E (C-term)	99.9
8	131R (TMIII) – 417E (C-term)	16.9	130D (TMIII) – 381R ($\alpha 4$ - $\beta 6$)	0.2
9	131R (TMIII) – 419L (C-term)	14.4	131R (TMIII) – 381R ($\alpha 4$ - $\beta 6$)	0.1
10	141Y (I2) – 410R ($\alpha 5$)	0.2	134A (TMIII) – 381R ($\alpha 4$ - $\beta 6$)	6.2
11	219Y (TMV) – 419L (C-term)	0.4	135I (TMIII) – 381R ($\alpha 4$ - $\beta 6$)	0.2
12	226A (I3) – 414R (C-term)	0.2	136T (I2) – 379D ($\alpha 4$ - $\beta 6$)	91.5
13	229Q (I3) – 406D ($\alpha 5$)	0.5	137S (I2) – 379D ($\alpha 4$ - $\beta 6$)	94.4
14	229Q (I3) – 410R ($\alpha 5$)	9.7	140K (I2) – 376A ($\alpha 4$)	46.3
15	229Q (I3) – 414R (C-term)	3.3	141Y (I2) – 302W ($\alpha 3$)	1.3
16	237E (I3) – 403D ($\alpha 5$)	65.6	141Y (I2) – 379D ($\alpha 4$ - $\beta 6$)	86.9
17	263K (I3) – 311S ($\alpha 3$ - $\beta 5$)	57.7	141Y (I2) – 380G ($\alpha 4$ - $\beta 6$)	0.2
18	263K (I3) – 312V ($\alpha 3$ - $\beta 5$)	0.2	141Y (I2) – 382H ($\alpha 4$ - $\beta 6$)	90.9
19	263K (I3) – 385Y ($\beta 6$)	2.5	143S (I2) – 383Y ($\alpha 4$ - $\beta 6$)	11.7
20	264F (I3) – 385Y ($\beta 6$)	95.4	145L (I2) – 383Y ($\alpha 4$ - $\beta 6$)	93.8
21	267K (I3) – 385Y ($\beta 6$)	1.2	145L (I2) – 414R (C-term)	0.2
22	267K (I3) – 414R (C-term)	0.2	225E (I3) – 308R ($\alpha 3$ - $\beta 5$)	86.3
23	267K (I3) – 415Q (C-term)	0.1	226A (I3) – 305R ($\alpha 3$)	94.9
24	270K (TMVI) – 380G ($\alpha 4$ - $\beta 6$)	2.4	227K (I3) – 305R ($\alpha 3$)	0.1
25	270K (TMVI) – 381R ($\alpha 4$ - $\beta 6$)	0.1	229Q (I3) – 303N ($\alpha 3$)	0.1
26	273K (TMVI) – 379D ($\alpha 4$ - $\beta 6$)	24.6	229Q (I3) – 305R ($\alpha 3$)	2.3
27	274T (TMVI) – 417E (C-term)	0.1	229Q (I3) – 308R ($\alpha 3$ - $\beta 5$)	27.6
28	328R (C-term) – 381R ($\alpha 4$ - $\beta 6$)	0.1	263K (I3) – 264N ($\alpha 2$ - $\beta 4$)	10.9
29	329S (C-term) – 381R ($\alpha 4$ - $\beta 6$)	3.0	264F (I3) – 305R ($\alpha 3$)	0.2
30	331D (C-term) – 308R ($\alpha 3$ - $\beta 5$)	65.1	267K (I3) – 264N ($\alpha 2$ - $\beta 4$)	9.7
31	331D (C-term) – 309T ($\alpha 3$ - $\beta 5$)	99.7	268E (TMVI) – 305R ($\alpha 3$)	67.2
32	331D (C-term) – 310I ($\alpha 3$ - $\beta 5$)	60.6	332F (C-term) – 416Y (C-term)	94.6
33	331D (C-term) – 415Q (C-term)	98.8	333R (C-term) – 417E (C-term)	96.0
34	333R (C-term) – 417E (C-term)	5.3	333R (C-term) – 419L (C-term)	0.1
35	335A (C-term) – 308R ($\alpha 3$ - $\beta 5$)	0.1	336F (C-term) – 416Y (C-term)	8.2
36	338E (C-term) – 305R ($\alpha 3$)	67.0	338E (C-term) – 56R (N-term)	0.2
37	338E (C-term) – 308R ($\alpha 3$ - $\beta 5$)	18.5	346S (C-term) – 53G (N-term)	6.0
38	346S (C-term) – 304 N ($\alpha 3$)	0.1	346S (C-term) – 56R (N-term)	0.2

Figure captions

Fig. 1 Amino acid sequences

The amino acid sequences for h β_2 R and human G α_s are given. For the h β_2 R the highly conserved amino acids according [38] are given in bold. All amino acids, establishing an interaction during the productive phase of the MD simulation between h β_2 R and G α_s are given in colored boxes: green (model I), blue (model II), red (model I and II)

Fig. 2 Axis system for h β_2 R-G α_s -complex

Fig. 3 Calculated potential energy surfaces between h β_2 R and G α_s

A, inactive h β_2 R and G α_s ; z: variation of the distance between h β_2 R and G α_s ; γ : rotation of G α_s around the z axis. B, inactive h β_2 R and translation of G α_s in the xy-plane at distinct z, α , β and γ . C, active h β_2 R and G α_s ; z: variation of the distance between h β_2 R and G α_s ; γ : rotation of G α_s around the z axis. D, active h β_2 R and translation of G α_s in the xy-plane at distinct z, α , β and γ . E, z: variation of the distance between h β_2 R and G α_s ; γ : rotation of G α_s around the z axis; distinct x, y, α and β ; red: areas with the active-h β_2 R-G α_s -complex being energetically preferred; blue: areas with the inactive-h β_2 R-G α_s -complex being energetically preferred. F, Potential energy for the inactive-h β_2 R-G α_s - (blue) and active-h β_2 R-G α_s -(red)-complex, according to model I and active h β_2 R-G α_s -(grey)-complex, according to model II, in dependence of variation of distance z between receptor and G α -subunit

Fig. 4 Two schematic models for preferred receptor-G-protein-interaction

Model I, the G $\beta\gamma$ -interaction site of the G α is located between TM VI and TM VII of the receptor. In this model, a long C-Terminus may interact with the G $\beta\gamma$ subunit and thus stabilize the receptor-G-protein-complex. Model II, the G $\beta\gamma$ -interaction site of the G α_s is located between TM V and TM VI. Thus, in this model a long I2-loop may interact with the G $\beta\gamma$ subunit in order to stabilize the receptor-G-protein-complex

Fig. 5 Hydrogen bond interactions between h β_2 R and G α_s

A, number of hydrogen bonds during the 2.5 ns productive phase of the molecular dynamics simulation of the inactive h β_2 R-G α_s -complex. B, number of hydrogen bonds during the 2.5 ns productive phase of the molecular dynamics simulations of the active h β_2 R-G α_s -complex, according to model I. C, distinct hydrogen bond interactions between the active h β_2 R and the G α_s during the 2.5 ns productive phase of molecular dynamics, according model I (1:63R-264N, 2:63R-265D, 3:63R-415Q, 4:66T-417E, 5:68T-416Y, 6:68T-417E, 7:69N-417E, 8:131R-417E, 9:131R-419L, 10:141Y-410R, 11:219Y-419L, 12:226A-414R, 13:229Q-406D, 14:229Q-410R, 15:229Q-414R, 16:237E-403D, 17:263K-311S, 18:263K-312V, 19:263K-385Y, 20:264F-385Y, 21:267K-385Y, 22:267K-414R, 23:267K-415Q, 24:270K-380G, 25:270K-381R, 26:273K-379D, 27:274T-417E, 28:328R-381R, 29:329S-381R, 30:331D-308R, 31:331D-309T, 32:331D-310I, 33:285D-415Q, 34:333R-417E, 35:335A-308R, 36:338E-305R, 37:338E-308R, 38:346S-304N). D, number of hydrogen bonds during the 2.5 ns productive phase of the molecular dynamics simulations of the active h β_2 R-G α_s -complex, according to model II. E, distinct hydrogen bond interactions between the active h β_2 R and the G α_s during the 2.5 ns productive phase of molecular dynamics, according model II (1:63R-409Q, 2:63R-412H, 3:63R-66E, 4:66T-414R, 5:68T-414R, 6:68T-418L, 7:69N-417E, 8:130D-381R, 9:131R-381R, 10:134A-381R, 11:135I-381R, 12:136T-379D, 13:137S-379D, 14:140K-376A, 15:141Y-302W, 16:141Y-379D, 17:141Y-380G, 18:141Y-382H, 19:143S-383Y, 20:145L-383Y, 21:145L-414R, 22:225E-308R, 23:226A-305R, 24:227K-305R, 25:229Q-303N, 26:229Q-305R, 27:229Q-308R, 28:263K-264N, 29:264F-305R, 30:267K-264N, 31:268E-305R, 32:332F-416Y, 33:333R-417E, 34:333R-419L, 35:336F-416Y, 36:338E-56R, 37:346S-53G, 38:346S-56R)

Fig. 6 Scheme for interactions between receptor and G α subunit, according to model I and II

Predicted interactions between receptor and G α subunit, based on the interactions detected for both energetically favored h β_2 R-G α_s complexes. Only the parts with more than 50 % hydrogen bond interaction during the productive phase between at least 2 amino acids are included

Fig. 7 Mean number of amino acids of I3-loop or C-terminus of human aminergic GPCRs

The mean number of amino acids of I3-loop or C-terminus of all human aminergic amino acids. The calculation was divided into receptors preferentially interacting with $G\alpha_s$, $G\alpha_i$ or $G\alpha_q$. The data shown are mean $\pm$ S.E.M

Figure 1
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hβ₂R

|-----N-terminus-----| |-----TMI-----| |---I1---|-----TMII---
 1-MGQPGNGSAFLLAPNGSHAPDHDVTQQRDEVWVVGMGIVMSLIVLAIVFGNVLVITAIKFE¹LQ²V³MYFITS⁴LACADLV⁵MG-83
 -----| |---E1---| |-----TMIII-----| |---I2---| |-----TMIV-----
 84-LAVVPFGAAHILMKMWTFGNFWCEFWTSLDVLCVTAS⁶IETLCVIAV⁷RYF⁸Y⁹Y¹⁰PF¹¹LQ¹²L¹³TKNKARV¹⁴IILMV¹⁵WIVSGLTSF-166
 ---| |-----E2-----| |-----TMV-----| |---I3---
 167-LPIQMHWYRATHQEAINCYANETCCDFFTNQAYAIASSIVSFYVPLVIMVFV¹⁶Y¹⁷SRVFQ¹⁸Y¹⁹Y²⁰LQKIDKSE²¹GRFHVQNLSQVE-249
 -----| |-----TMVI-----| |---E3---| |-----TMVII-----| |-----
 250-QDGR²²TGHGLRRSS²³Y²⁴CL²⁵Y²⁶HKAL²⁷KT²⁸LGII²⁹MGTF³⁰TL³¹CWL³²PFFIVNIVHVIQDN³³LIRKEVYILLNWIGYVNSGFNPLIYCR³⁴SPD³⁵-332
 -----C-terminus-----|
 333-Y³⁶IA³⁷Q³⁸LLCLRRS³⁹Y⁴⁰LKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVP⁴¹SDNIDSPGRNCSTNDSL⁴²L-413

hGα_s

|-----N-terminus-----| |αN-β1| |β1-|
 1-PVRSSAPRRGHSVASAPRSGLRQVAGRRGAALPCSLAPGCGAAAGASPCPGA¹RR²Y³AAGGRCLAC⁴CTSLTCAGESGKSTIVK-83
 84-QMRILHVNGFN⁵GEGGEEDPQAARSNSDGEKATKVQDIKNNLKEA⁶IETIVAAMSNLVPPVELANPENQFRVDYILSVMNVPDFD-166
 167-FPPEFYEHAKALWEDEGVRACYERSNEYQLIDCAQYFLDKIDVIKQADYVPSDQDLLRCRVLTSGIFETKFQVDKVN⁷FHMFDV-249
 |---α2---| |α2-β4---| |---β4---| |---SwIII---| |---α3---| |α3-β5| |---β5---| |-----
 250-GGQDERRKWIQCF⁸Y⁹VT¹⁰TAII¹¹FVVASSYNMVIREDNQT¹²NRLQEALNLFKSI¹³Y¹⁴Y¹⁵Y¹⁶Y¹⁷Y¹⁸Y¹⁹Y²⁰Y²¹Y²²Y²³Y²⁴Y²⁵Y²⁶Y²⁷Y²⁸Y²⁹Y³⁰Y³¹Y³²Y³³Y³⁴Y³⁵Y³⁶Y³⁷Y³⁸Y³⁹Y⁴⁰Y⁴¹Y⁴²Y⁴³Y⁴⁴Y⁴⁵Y⁴⁶Y⁴⁷Y⁴⁸Y⁴⁹Y⁵⁰Y⁵¹Y⁵²Y⁵³Y⁵⁴Y⁵⁵Y⁵⁶Y⁵⁷Y⁵⁸Y⁵⁹Y⁶⁰Y⁶¹Y⁶²Y⁶³Y⁶⁴Y⁶⁵Y⁶⁶Y⁶⁷Y⁶⁸Y⁶⁹Y⁷⁰Y⁷¹Y⁷²Y⁷³Y⁷⁴Y⁷⁵Y⁷⁶Y⁷⁷Y⁷⁸Y⁷⁹Y⁸⁰Y⁸¹Y⁸²Y⁸³Y⁸⁴Y⁸⁵Y⁸⁶Y⁸⁷Y⁸⁸Y⁸⁹Y⁹⁰Y⁹¹Y⁹²Y⁹³Y⁹⁴Y⁹⁵Y⁹⁶Y⁹⁷Y⁹⁸Y⁹⁹Y¹⁰⁰Y¹⁰¹Y¹⁰²Y¹⁰³Y¹⁰⁴Y¹⁰⁵Y¹⁰⁶Y¹⁰⁷Y¹⁰⁸Y¹⁰⁹Y¹¹⁰Y¹¹¹Y¹¹²Y¹¹³Y¹¹⁴Y¹¹⁵Y¹¹⁶Y¹¹⁷Y¹¹⁸Y¹¹⁹Y¹²⁰Y¹²¹Y¹²²Y¹²³Y¹²⁴Y¹²⁵Y¹²⁶Y¹²⁷Y¹²⁸Y¹²⁹Y¹³⁰Y¹³¹Y¹³²Y¹³³Y¹³⁴Y¹³⁵Y¹³⁶Y¹³⁷Y¹³⁸Y¹³⁹Y¹⁴⁰Y¹⁴¹Y¹⁴²Y¹⁴³Y¹⁴⁴Y¹⁴⁵Y¹⁴⁶Y¹⁴⁷Y¹⁴⁸Y¹⁴⁹Y¹⁵⁰Y¹⁵¹Y¹⁵²Y¹⁵³Y¹⁵⁴Y¹⁵⁵Y¹⁵⁶Y¹⁵⁷Y¹⁵⁸Y¹⁵⁹Y¹⁶⁰Y¹⁶¹Y¹⁶²Y¹⁶³Y¹⁶⁴Y¹⁶⁵Y¹⁶⁶Y¹⁶⁷Y¹⁶⁸Y¹⁶⁹Y¹⁷⁰Y¹⁷¹Y¹⁷²Y¹⁷³Y¹⁷⁴Y¹⁷⁵Y¹⁷⁶Y¹⁷⁷Y¹⁷⁸Y¹⁷⁹Y¹⁸⁰Y¹⁸¹Y¹⁸²Y¹⁸³Y¹⁸⁴Y¹⁸⁵Y¹⁸⁶Y¹⁸⁷Y¹⁸⁸Y¹⁸⁹Y¹⁹⁰Y¹⁹¹Y¹⁹²Y¹⁹³Y¹⁹⁴Y¹⁹⁵Y¹⁹⁶Y¹⁹⁷Y¹⁹⁸Y¹⁹⁹Y²⁰⁰Y²⁰¹Y²⁰²Y²⁰³Y²⁰⁴Y²⁰⁵Y²⁰⁶Y²⁰⁷Y²⁰⁸Y²⁰⁹Y²¹⁰Y²¹¹Y²¹²Y²¹³Y²¹⁴Y²¹⁵Y²¹⁶Y²¹⁷Y²¹⁸Y²¹⁹Y²²⁰Y²²¹Y²²²Y²²³Y²²⁴Y²²⁵Y²²⁶Y²²⁷Y²²⁸Y²²⁹Y²³⁰Y²³¹Y²³²Y²³³Y²³⁴Y²³⁵Y²³⁶Y²³⁷Y²³⁸Y²³⁹Y²⁴⁰Y²⁴¹Y²⁴²Y²⁴³Y²⁴⁴Y²⁴⁵Y²⁴⁶Y²⁴⁷Y²⁴⁸Y²⁴⁹Y²⁵⁰Y²⁵¹Y²⁵²Y²⁵³Y²⁵⁴Y²⁵⁵Y²⁵⁶Y²⁵⁷Y²⁵⁸Y²⁵⁹Y²⁶⁰Y²⁶¹Y²⁶²Y²⁶³Y²⁶⁴Y²⁶⁵Y²⁶⁶Y²⁶⁷Y²⁶⁸Y²⁶⁹Y²⁷⁰Y²⁷¹Y²⁷²Y²⁷³Y²⁷⁴Y²⁷⁵Y²⁷⁶Y²⁷⁷Y²⁷⁸Y²⁷⁹Y²⁸⁰Y²⁸¹Y²⁸²Y²⁸³Y²⁸⁴Y²⁸⁵Y²⁸⁶Y²⁸⁷Y²⁸⁸Y²⁸⁹Y²⁹⁰Y²⁹¹Y²⁹²Y²⁹³Y²⁹⁴Y²⁹⁵Y²⁹⁶Y²⁹⁷Y²⁹⁸Y²⁹⁹Y³⁰⁰Y³⁰¹Y³⁰²Y³⁰³Y³⁰⁴Y³⁰⁵Y³⁰⁶Y³⁰⁷Y³⁰⁸Y³⁰⁹Y³¹⁰Y³¹¹Y³¹²Y³¹³Y³¹⁴Y³¹⁵Y³¹⁶Y³¹⁷Y³¹⁸Y³¹⁹Y³²⁰Y³²¹Y³²²Y³²³Y³²⁴Y³²⁵Y³²⁶Y³²⁷Y³²⁸Y³²⁹Y³³⁰Y³³¹Y³³²Y³³³Y³³⁴Y³³⁵Y³³⁶Y³³⁷Y³³⁸Y³³⁹Y³⁴⁰Y³⁴¹Y³⁴²Y³⁴³Y³⁴⁴Y³⁴⁵Y³⁴⁶Y³⁴⁷Y³⁴⁸Y³⁴⁹Y³⁵⁰Y³⁵¹Y³⁵²Y³⁵³Y³⁵⁴Y³⁵⁵Y³⁵⁶Y³⁵⁷Y³⁵⁸Y³⁵⁹Y³⁶⁰Y³⁶¹Y³⁶²Y³⁶³Y³⁶⁴Y³⁶⁵Y³⁶⁶Y³⁶⁷Y³⁶⁸Y³⁶⁹Y³⁷⁰Y³⁷¹Y³⁷²Y³⁷³Y³⁷⁴Y³⁷⁵Y³⁷⁶Y³⁷⁷Y³⁷⁸Y³⁷⁹Y³⁸⁰Y³⁸¹Y³⁸²Y³⁸³Y³⁸⁴Y³⁸⁵Y³⁸⁶Y³⁸⁷Y³⁸⁸Y³⁸⁹Y³⁹⁰Y³⁹¹Y³⁹²Y³⁹³Y³⁹⁴Y³⁹⁵Y³⁹⁶Y³⁹⁷Y³⁹⁸Y³⁹⁹Y⁴⁰⁰Y⁴⁰¹Y⁴⁰²Y⁴⁰³Y⁴⁰⁴Y⁴⁰⁵Y⁴⁰⁶Y⁴⁰⁷Y⁴⁰⁸Y⁴⁰⁹Y⁴¹⁰Y⁴¹¹Y⁴¹²Y⁴¹³Y⁴¹⁴Y⁴¹⁵Y⁴¹⁶Y⁴¹⁷Y⁴¹⁸Y⁴¹⁹Y⁴²⁰Y⁴²¹Y⁴²²Y⁴²³Y⁴²⁴Y⁴²⁵Y⁴²⁶Y⁴²⁷Y⁴²⁸Y⁴²⁹Y⁴³⁰Y⁴³¹Y⁴³²Y⁴³³Y⁴³⁴Y⁴³⁵Y⁴³⁶Y⁴³⁷Y⁴³⁸Y⁴³⁹Y⁴⁴⁰Y⁴⁴¹Y⁴⁴²Y⁴⁴³Y⁴⁴⁴Y⁴⁴⁵Y⁴⁴⁶Y⁴⁴⁷Y⁴⁴⁸Y⁴⁴⁹Y⁴⁵⁰Y⁴⁵¹Y⁴⁵²Y⁴⁵³Y⁴⁵⁴Y⁴⁵⁵Y⁴⁵⁶Y⁴⁵⁷Y⁴⁵⁸Y⁴⁵⁹Y⁴⁶⁰Y⁴⁶¹Y⁴⁶²Y⁴⁶³Y⁴⁶⁴Y⁴⁶⁵Y⁴⁶⁶Y⁴⁶⁷Y⁴⁶⁸Y⁴⁶⁹Y⁴⁷⁰Y⁴⁷¹Y⁴⁷²Y⁴⁷³Y⁴⁷⁴Y⁴⁷⁵Y⁴⁷⁶Y⁴⁷⁷Y⁴⁷⁸Y⁴⁷⁹Y⁴⁸⁰Y⁴⁸¹Y⁴⁸²Y⁴⁸³Y⁴⁸⁴Y⁴⁸⁵Y⁴⁸⁶Y⁴⁸⁷Y⁴⁸⁸Y⁴⁸⁹Y⁴⁹⁰Y⁴⁹¹Y⁴⁹²Y⁴⁹³Y⁴⁹⁴Y⁴⁹⁵Y⁴⁹⁶Y⁴⁹⁷Y⁴⁹⁸Y⁴⁹⁹Y⁵⁰⁰Y⁵⁰¹Y⁵⁰²Y⁵⁰³Y⁵⁰⁴Y⁵⁰⁵Y⁵⁰⁶Y⁵⁰⁷Y⁵⁰⁸Y⁵⁰⁹Y⁵¹⁰Y⁵¹¹Y⁵¹²Y⁵¹³Y⁵¹⁴Y⁵¹⁵Y⁵¹⁶Y⁵¹⁷Y⁵¹⁸Y⁵¹⁹Y⁵²⁰Y⁵²¹Y⁵²²Y⁵²³Y⁵²⁴Y⁵²⁵Y⁵²⁶Y⁵²⁷Y⁵²⁸Y⁵²⁹Y⁵³⁰Y⁵³¹Y⁵³²Y⁵³³Y⁵³⁴Y⁵³⁵Y⁵³⁶Y⁵³⁷Y⁵³⁸Y⁵³⁹Y⁵⁴⁰Y⁵⁴¹Y⁵⁴²Y⁵⁴³Y⁵⁴⁴Y⁵⁴⁵Y⁵⁴⁶Y⁵⁴⁷Y⁵⁴⁸Y⁵⁴⁹Y⁵⁵⁰Y⁵⁵¹Y⁵⁵²Y⁵⁵³Y⁵⁵⁴Y⁵⁵⁵Y⁵⁵⁶Y⁵⁵⁷Y⁵⁵⁸Y⁵⁵⁹Y⁵⁶⁰Y⁵⁶¹Y⁵⁶²Y⁵⁶³Y⁵⁶⁴Y⁵⁶⁵Y⁵⁶⁶Y⁵⁶⁷Y⁵⁶⁸Y⁵⁶⁹Y⁵⁷⁰Y⁵⁷¹Y⁵⁷²Y⁵⁷³Y⁵⁷⁴Y⁵⁷⁵Y⁵⁷⁶Y⁵⁷⁷Y⁵⁷⁸Y⁵⁷⁹Y⁵⁸⁰Y⁵⁸¹Y⁵⁸²Y⁵⁸³Y⁵⁸⁴Y⁵⁸⁵Y⁵⁸⁶Y⁵⁸⁷Y⁵⁸⁸Y⁵⁸⁹Y⁵⁹⁰Y⁵⁹¹Y⁵⁹²Y⁵⁹³Y⁵⁹⁴Y⁵⁹⁵Y⁵⁹⁶Y⁵⁹⁷Y⁵⁹⁸Y⁵⁹⁹Y⁶⁰⁰Y⁶⁰¹Y⁶⁰²Y⁶⁰³Y⁶⁰⁴Y⁶⁰⁵Y⁶⁰⁶Y⁶⁰⁷Y⁶⁰⁸Y⁶⁰⁹Y⁶¹⁰Y⁶¹¹Y⁶¹²Y⁶¹³Y⁶¹⁴Y⁶¹⁵Y⁶¹⁶Y⁶¹⁷Y⁶¹⁸Y⁶¹⁹Y⁶²⁰Y⁶²¹Y⁶²²Y⁶²³Y⁶²⁴Y⁶²⁵Y⁶²⁶Y⁶²⁷Y⁶²⁸Y⁶²⁹Y⁶³⁰Y⁶³¹Y⁶³²Y⁶³³Y⁶³⁴Y⁶³⁵Y⁶³⁶Y⁶³⁷Y⁶³⁸Y⁶³⁹Y⁶⁴⁰Y⁶⁴¹Y⁶⁴²Y⁶⁴³Y⁶⁴⁴Y⁶⁴⁵Y⁶⁴⁶Y⁶⁴⁷Y⁶⁴⁸Y⁶⁴⁹Y⁶⁵⁰Y⁶⁵¹Y⁶⁵²Y⁶⁵³Y⁶⁵⁴Y⁶⁵⁵Y⁶⁵⁶Y⁶⁵⁷Y⁶⁵⁸Y⁶⁵⁹Y⁶⁶⁰Y⁶⁶¹Y⁶⁶²Y⁶⁶³Y⁶⁶⁴Y⁶⁶⁵Y⁶⁶⁶Y⁶⁶⁷Y⁶⁶⁸Y⁶⁶⁹Y⁶⁷⁰Y⁶⁷¹Y⁶⁷²Y⁶⁷³Y⁶⁷⁴Y⁶⁷⁵Y⁶⁷⁶Y⁶⁷⁷Y⁶⁷⁸Y⁶⁷⁹Y⁶⁸⁰Y⁶⁸¹Y⁶⁸²Y⁶⁸³Y⁶⁸⁴Y⁶⁸⁵Y⁶⁸⁶Y⁶⁸⁷Y⁶⁸⁸Y⁶⁸⁹Y⁶⁹⁰Y⁶⁹¹Y⁶⁹²Y⁶⁹³Y⁶⁹⁴Y⁶⁹⁵Y⁶⁹⁶Y⁶⁹⁷Y⁶⁹⁸Y⁶⁹⁹Y⁷⁰⁰Y⁷⁰¹Y⁷⁰²Y⁷⁰³Y⁷⁰⁴Y⁷⁰⁵Y⁷⁰⁶Y⁷⁰⁷Y⁷⁰⁸Y⁷⁰⁹Y⁷¹⁰Y⁷¹¹Y⁷¹²Y⁷¹³Y⁷¹⁴Y⁷¹⁵Y⁷¹⁶Y⁷¹⁷Y⁷¹⁸Y⁷¹⁹Y⁷²⁰Y⁷²¹Y⁷²²Y⁷²³Y⁷²⁴Y⁷²⁵Y⁷²⁶Y⁷²⁷Y⁷²⁸Y⁷²⁹Y⁷³⁰Y⁷³¹Y⁷³²Y⁷³³Y⁷³⁴Y⁷³⁵Y⁷³⁶Y⁷³⁷Y⁷³⁸Y⁷³⁹Y⁷⁴⁰Y⁷⁴¹Y⁷⁴²Y⁷⁴³Y⁷⁴⁴Y⁷⁴⁵Y⁷⁴⁶Y⁷⁴⁷Y⁷⁴⁸Y⁷⁴⁹Y⁷⁵⁰Y⁷⁵¹Y⁷⁵²Y⁷⁵³Y⁷⁵⁴Y⁷⁵⁵Y⁷⁵⁶Y⁷⁵⁷Y⁷⁵⁸Y⁷⁵⁹Y⁷⁶⁰Y⁷⁶¹Y⁷⁶²Y⁷⁶³Y⁷⁶⁴Y⁷⁶⁵Y⁷⁶⁶Y⁷⁶⁷Y⁷⁶⁸Y⁷⁶⁹Y⁷⁷⁰Y⁷⁷¹Y⁷⁷²Y⁷⁷³Y⁷⁷⁴Y⁷⁷⁵Y⁷⁷⁶Y⁷⁷⁷Y⁷⁷⁸Y⁷⁷⁹Y⁷⁸⁰Y⁷⁸¹Y⁷⁸²Y⁷⁸³Y⁷⁸⁴Y⁷⁸⁵Y⁷⁸⁶Y⁷⁸⁷Y⁷⁸⁸Y⁷⁸⁹Y⁷⁹⁰Y⁷⁹¹Y⁷⁹²Y⁷⁹³Y⁷⁹⁴Y⁷⁹⁵Y⁷⁹⁶Y⁷⁹⁷Y⁷⁹⁸Y⁷⁹⁹Y⁸⁰⁰Y⁸⁰¹Y⁸⁰²Y⁸⁰³Y⁸⁰⁴Y⁸⁰⁵Y⁸⁰⁶Y⁸⁰⁷Y⁸⁰⁸Y⁸⁰⁹Y⁸¹⁰Y⁸¹¹Y⁸¹²Y⁸¹³Y⁸¹⁴Y⁸¹⁵Y⁸¹⁶Y⁸¹⁷Y⁸¹⁸Y⁸¹⁹Y⁸²⁰Y⁸²¹Y⁸²²Y⁸²³Y⁸²⁴Y⁸²⁵Y⁸²⁶Y⁸²⁷Y⁸²⁸Y⁸²⁹Y⁸³⁰Y⁸³¹Y⁸³²Y⁸³³Y⁸³⁴Y⁸³⁵Y⁸³⁶Y⁸³⁷Y⁸³⁸Y⁸³⁹Y⁸⁴⁰Y⁸⁴¹Y⁸⁴²Y⁸⁴³Y⁸⁴⁴Y⁸⁴⁵Y⁸⁴⁶Y⁸⁴⁷Y⁸⁴⁸Y⁸⁴⁹Y⁸⁵⁰Y⁸⁵¹Y⁸⁵²Y⁸⁵³Y⁸⁵⁴Y⁸⁵⁵Y⁸⁵⁶Y⁸⁵⁷Y⁸⁵⁸Y⁸⁵⁹Y⁸⁶⁰Y⁸⁶¹Y⁸⁶²Y⁸⁶³Y⁸⁶⁴Y⁸⁶⁵Y⁸⁶⁶Y⁸⁶⁷Y⁸⁶⁸Y⁸⁶⁹Y⁸⁷⁰Y⁸⁷¹Y⁸⁷²Y⁸⁷³Y⁸⁷⁴Y⁸⁷⁵Y⁸⁷⁶Y⁸⁷⁷Y⁸⁷⁸Y⁸⁷⁹Y⁸⁸⁰Y⁸⁸¹Y⁸⁸²Y⁸⁸³Y⁸⁸⁴Y⁸⁸⁵Y⁸⁸⁶Y⁸⁸⁷Y⁸⁸⁸Y⁸⁸⁹Y⁸⁹⁰Y⁸⁹¹Y⁸⁹²Y⁸⁹³Y⁸⁹⁴Y⁸⁹⁵Y⁸⁹⁶Y⁸⁹⁷Y⁸⁹⁸Y⁸⁹⁹Y⁹⁰⁰Y⁹⁰¹Y⁹⁰²Y⁹⁰³Y⁹⁰⁴Y⁹⁰⁵Y⁹⁰⁶Y⁹⁰⁷Y⁹⁰⁸Y⁹⁰⁹Y⁹¹⁰Y⁹¹¹Y⁹¹²Y⁹¹³Y⁹¹⁴Y⁹¹⁵Y⁹¹⁶Y⁹¹⁷Y⁹¹⁸Y⁹¹⁹Y⁹²⁰Y⁹²¹Y⁹²²Y⁹²³Y⁹²⁴Y⁹²⁵Y⁹²⁶Y⁹²⁷Y⁹²⁸Y⁹²⁹Y⁹³⁰Y⁹³¹Y⁹³²Y⁹³³Y⁹³⁴Y⁹³⁵Y⁹³⁶Y⁹³⁷Y⁹³⁸Y⁹³⁹Y⁹⁴⁰Y⁹⁴¹Y⁹⁴²Y⁹⁴³Y⁹⁴⁴Y⁹⁴⁵Y⁹⁴⁶Y⁹⁴⁷Y⁹⁴⁸Y⁹⁴⁹Y⁹⁵⁰Y⁹⁵¹Y⁹⁵²Y⁹⁵³Y⁹⁵⁴Y⁹⁵⁵Y⁹⁵⁶Y⁹⁵⁷Y⁹⁵⁸Y⁹⁵⁹Y⁹⁶⁰Y⁹⁶¹Y⁹⁶²Y⁹⁶³Y⁹⁶⁴Y⁹⁶⁵Y⁹⁶⁶Y⁹⁶⁷Y⁹⁶⁸Y⁹⁶⁹Y⁹⁷⁰Y⁹⁷¹Y⁹⁷²Y⁹⁷³Y⁹⁷⁴Y⁹⁷⁵Y⁹⁷⁶Y⁹⁷⁷Y⁹⁷⁸Y⁹⁷⁹Y⁹⁸⁰Y⁹⁸¹Y⁹⁸²Y⁹⁸³Y⁹⁸⁴Y⁹⁸⁵Y⁹⁸⁶Y⁹⁸⁷Y⁹⁸⁸Y⁹⁸⁹Y⁹⁹⁰Y⁹⁹¹Y⁹⁹²Y⁹⁹³Y⁹⁹⁴Y⁹⁹⁵Y⁹⁹⁶Y⁹⁹⁷Y⁹⁹⁸Y⁹⁹⁹Y¹⁰⁰⁰Y¹⁰⁰¹Y¹⁰⁰²Y¹⁰⁰³Y¹⁰⁰⁴Y¹⁰⁰⁵Y¹⁰⁰⁶Y¹⁰⁰⁷Y¹⁰⁰⁸Y¹⁰⁰⁹Y¹⁰¹⁰Y¹⁰¹¹Y¹⁰¹²Y¹⁰¹³Y¹⁰¹⁴Y¹⁰¹⁵Y¹⁰¹⁶Y¹⁰¹⁷Y¹⁰¹⁸Y¹⁰¹⁹Y¹⁰²⁰Y¹⁰²¹Y¹⁰²²Y¹⁰²³Y¹⁰²⁴Y¹⁰²⁵Y¹⁰²⁶Y¹⁰²⁷Y¹⁰²⁸Y¹⁰²⁹Y¹⁰³⁰Y¹⁰³¹Y¹⁰³²Y¹⁰³³Y¹⁰³⁴Y¹⁰³⁵Y¹⁰³⁶Y¹⁰³⁷Y¹⁰³⁸Y¹⁰³⁹Y¹⁰⁴⁰Y¹⁰⁴¹Y¹⁰⁴²Y¹⁰⁴³Y¹⁰⁴⁴Y¹⁰⁴⁵Y¹⁰⁴⁶Y¹⁰⁴⁷Y¹⁰⁴⁸Y¹⁰⁴⁹Y¹⁰⁵⁰Y¹⁰⁵¹Y¹⁰⁵²Y¹⁰⁵³Y¹⁰⁵⁴Y¹⁰⁵⁵Y¹⁰⁵⁶Y¹⁰⁵⁷Y¹⁰⁵⁸Y¹⁰⁵⁹Y¹⁰⁶⁰Y¹⁰⁶¹Y¹⁰⁶²Y¹⁰⁶³Y¹⁰⁶⁴Y¹⁰⁶⁵Y¹⁰⁶⁶Y¹⁰⁶⁷Y¹⁰⁶⁸Y¹⁰⁶⁹Y¹⁰⁷⁰Y¹⁰⁷¹Y¹⁰⁷²Y¹⁰⁷³Y¹⁰⁷⁴Y¹⁰⁷⁵Y¹⁰⁷⁶Y¹⁰⁷⁷Y¹⁰⁷⁸Y¹⁰⁷⁹Y¹⁰⁸⁰Y¹⁰⁸¹Y¹⁰⁸²Y¹⁰⁸³Y¹⁰⁸⁴Y¹⁰⁸⁵Y¹⁰⁸⁶Y¹⁰⁸⁷Y¹⁰⁸⁸Y¹⁰⁸⁹Y¹⁰⁹⁰Y¹⁰⁹¹Y¹⁰⁹²Y¹⁰⁹³Y¹⁰⁹⁴Y¹⁰⁹⁵Y¹⁰⁹⁶Y¹⁰⁹⁷Y¹⁰⁹⁸Y¹⁰⁹⁹Y¹¹⁰⁰Y¹¹⁰¹Y¹¹⁰²Y¹¹⁰³Y¹¹⁰⁴Y¹¹⁰⁵Y¹¹⁰⁶Y¹¹⁰⁷Y¹¹⁰⁸Y¹¹⁰⁹Y¹¹¹⁰Y¹¹¹¹Y¹¹¹²Y¹¹¹³Y¹¹¹⁴Y¹¹¹⁵Y¹¹¹⁶Y^{1117</}

Figure 2
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