The mammalian olfactory system comprises a large family of G protein-coupled receptors (GPCRs) to detect and discriminate numerous volatile ligands. More than 350 human genes encode functional olfactory receptors (ORs) that belong to the class A (rhodopsin-like) GPCR family. Owing to difficulties with functional OR expression in heterologous systems, only a few human ORs have been characterized to date. Most deorphanized ORs, that is, ORs with a known ligand spectrum, detect multiple chemically similar odorants, and hypervariable residues in the seven transmembrane (7TM) helices (I–VII) have been postulated to form the basis for ligand specificity. A prerequisite for understanding olfactory receptor selectivity is information on the spatial properties of the ligand-binding niche. Different classes of approaches have been employed for such an assessment. Ligand-based approaches, such as pharmacophore modeling or quantitative structure-activity relationship (QSAR), can give valuable models of the ligand structure, which is required for discriminating activating and inactive ligands, and information on the form of the binding pocket. Receptor-based approaches such as homology modeling create a model of the protein and the binding site explicitly and from this give information on ligand binding. Both techniques can be combined together. For such receptor-based and mixed approaches, the X-ray structures of seven GPCRs have been solved to date, but none for ORs. Previous studies have used static structural models of different ORs based on a rhodopsin and a β2-adrenergic receptor (B2AR) template. However, most odorants are highly flexible, so assessment of the ligand/protein dynamics might be of crucial importance in understanding ligand recognition by ORs. To better understand receptor activation, we thus searched for a dynamic ligand–protein interaction pattern instead of analyzing ligand-binding in static models. Therefore, in difference to other flexible GPCR ligand pocket analysis approaches, we use the predictive power of protein/ligand complex molecular dynamics (MD) simulations to gain insight into the protein–odorant dynamics necessary for receptor activation.

We developed a dynamic model of the functionally well-characterized human olfactory receptor hOR2AG1. We used an X-ray structure of bovine rhodopsin with 2.2 Å resolution as starting structure for dynamic homology modeling of hOR2AG1, since both receptors belong to the class A GPCRs, and both harbor hydrophobic ligands. The performance of this approach was previously tested by homology modeling of the B2AR ligand-binding niche based on the rhodopsin template (see Supporting Information, Section I a). Although the overall sequence identity among class A GPCRs is relatively low, this can be compensated for by careful incorporation of experimental information as constraints. In the present study, site-directed mutagenesis and functional analysis of receptor mutants by Ca2+ imaging were performed for validation of the hOR2AG1 homology model. Combining both techniques in a cycle of dynamic computational predictions and experimental analysis based on site-directed mutagenesis, we were able to characterize and refine the three-dimensional structure of the...
characterization of recombinant receptors by single
Thr279.42 (Figure 1a; numbers in superscripts refers
analyzed the A104G, A104I, F206V, V260W, S263C, S263V,
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gen bonds as robust (present in 100–50% of the simulated
simulations, one with the ligand positioned horizontally and
putative binding cavity revealed two possible orienta-
tions, one with the ligand positioned horizontally and one
vertically to the membrane plane. For verification
indicated in blue, transmembrane helices (TMs) in gray. b) The effects of amino acid
mutations in TM III, V, VI, and VII on receptor activation as measured by Ca2+
images of Hana3a cells expressing hOR2AG1 variants. The bar chart illustrates relative receptor activation by amylbutyrate (600 μM). Mean receptor activation (response probability) was normalized to response probabilities of wild type (WT) hOR2AG1-expressing cells (black bar). Error bars indicate the standard errors of the mean (SEM). The data are representative of 15–40 independent experiments, each with 600–1600 cells. Significance according to Student’s t-test relative to the amylbutyrate response probability of WT hOR2AG1 (+P < 0.05, **P < 0.01, ***P < 0.001). Amylbutyrate application did not induce an increase in the cytosolic Ca2+ concentration in mock-transfected cells that was larger than the spontaneous activity induced solely by the application of Ringer’s solution (white bars). c) Computer model of hOR2AG1 (gray) with amylbutyrate (orange sticks). The ligand is bound between helices III, V, VI, and VII in a binding mode vertical to the membrane plane.

Simulations of the ligand amylbutyrate in the
putative binding cavity revealed two possible orienta-
tions, one with the ligand positioned horizontally and one
to Ballesteros–Weinstein numbering[44], which all form
contacts with the ligand in the vertical binding mode
during MD simulation (more details in Supporting
Information, Sections 2b and 2c). The experimental
analysis of point mutated receptors hOR2AG1-A104I,
-V260W, -S263V, -S264V, -S264C, and -T279V by Ca2+
imaging showed a decreased activity compared to the
WT receptor (Figure 1b and Table 1). Therefore, the
comparison of theoretical and experimental analysis
revealed the proposed cavity as amylbutyrate binding
cavity and amylbutyrate to be bound in a vertical
binding mode (Figure 1c).

A list of all putative ligand contact residues is
provided in the Supporting Information, Section 3a.
Ala1043.32, Phe2065.47, Val2606.48, Ser2636.51, Ser2646.52,
and Thr2797.42 are located on sequence positions that are highly
variable throughout the olfactory protein family and might
therefore determine ligand specificity[6,45,46] (Supporting
Information, Figure S3a, and Section 3b).

In addition, we experimentally analyzed four control
mutations, A104G, F206V, V239W, and S242C, which should
d not have any influence on ligand binding as judged by MD
simulations. In agreement with the model, in the experiment
none of the control mutations affected receptor activation
compared to the WT receptor (Figure 1b).

We investigated computationally if we could determine
a quantitative criterion for receptor activation. A dynamic
binding mode with fluctuating hydrogen bonds between
receptor and ligand offers a solution to cope with the high
flexibility of the odorant ligand.[47] Thus, we investigated the
hydrogen bond contact frequencies between amylbutyrate
and Ser2636.51, Ser2646.52, and Thr2797.42, by classifying hydro-
gen bonds as robust (present in 100–50% of the simulated
time), fluctuating (49–25%), and temporary (24–1%). We
analyzed the A104G, A104I, F206V, V260W, S263C, S263V,

<table>
<thead>
<tr>
<th>hOR2AG1 variant</th>
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<th>Activity rating[6]</th>
<th>In vivo activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Ser26351, Ser26452, Thr27942</td>
<td>active</td>
<td>active</td>
</tr>
<tr>
<td>A104G</td>
<td>12% 6% 92%</td>
<td>active</td>
<td>active</td>
</tr>
<tr>
<td>A104I</td>
<td>10% 8% 87%</td>
<td>active</td>
<td>active</td>
</tr>
<tr>
<td>F206V</td>
<td>3% 40% 61%</td>
<td>active</td>
<td>active</td>
</tr>
<tr>
<td>V260W</td>
<td>11% – 94%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S263C</td>
<td>2%[8]</td>
<td>2% 98%</td>
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<tr>
<td>S263V</td>
<td>41%</td>
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<td>S264V</td>
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<tr>
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[a] Temporary hydrogen bonding (> 0%) to Ser26351, Ser26452 and robust hydrogen bonding (> 49%) to Thr27942 were considered as activity criterion. [b] Mutation affects respective amino acid residue.

Table 1: Computed hydrogen-bond contacts of amylbutyrate with hOR2AG1 variants. The frequency of hydrogen-bond contact occurrence is shown as percentage of the 10 ns simulated time.

Figure 1. Characterization of the amylbutyrate-binding niche. a) Close-up of the putative ligand-binding niche of amylbutyrate bound to hOR2AG1 in the vertical binding mode after 10 ns free MD simulation. Ligand contact residues in blue, transmembrane helices (TMs) in gray. b) The effects of amino acid mutations in TM III, V, VI, and VII on receptor activation as measured by Ca2+ imaging[42,43] (see Supporting Information, Figure S1, and Section 2b). c) The putative ligand-binding cavity within a hOR2AG1 structural model to a new extent.

We first searched for a suitable ligand-binding
cavity in our model, which was verified by experimental
analysis. Simulations of the initial rhodopsin-based
hOR2AG1 model (for details in modeling and template
choice see Supporting Information, Section 1a) revealed a cavity between helices III, V, VI, and VII
as most promising ligand binding position (cavity A in Supporting
Information, Figure S1, and Section 2b). Experimental validation of the ligand binding cavity was carried out by expression of wild type (WT) and
mutant hOR2AG1 in Hana3a cells and functional
characterization of recombinant receptors by single
cell Ca2+ imaging[42,43] (see Supporting Information,
Figure S2, and Section 2a).

Table 2: Computed hydrogen-bond contacts of amylbutyrate with hOR2AG1 variants. The frequency of hydrogen-bond contact occurrence is shown as percentage of the 10 ns simulated time.

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[a] Temporary hydrogen bonding (> 0%) to Ser26351, Ser26452 and robust hydrogen bonding (> 49%) to Thr27942 were considered as activity criterion. [b] Mutation affects respective amino acid residue.

S264C, S264V, and T279V mutants in 10 ns free MD
simulations in the appropriate membrane/solvent environment (Supporting Information, Figure S4), comparing them
with the mutant receptor activation by amylbutyrate. In simulations of all mutant receptors, which were still functional in experimental analysis (see Figure 1b), Ser263\(^{51}\) and Ser264\(^{52}\) established at least temporary hydrogen bonds with the ligand (during 2–66% of simulation time, see Table 1), and Thr279\(^{42}\) showed a robust hydrogen bond (61–98% of simulated time, Table 1). The model also accounts for the remaining activity of the S263C mutant, as Cys263 still forms hydrogen bonds to amylbutyrate. We hypothesize that the dynamic hydrogen-bonding pattern of the ligand’s ester group to these three polar residues, especially Thr279\(^{42}\), serves as a criterion for receptor activation.

A similar binding mode of a protein with an acetyl ester can be found in the crystal structure of the Drosophila melanogaster odorant binding protein LUSH (protein data bank (PDB) code: 2GTE; 1.4 Å; see Supporting Information, Figure S5 and Section 4).\(^{[6]}\)

To further validate the proposed binding niche, we investigated if hydrogen bonds to Ser263\(^{51}\), Ser264\(^{52}\), and Thr279\(^{42}\) can be used to predict the binding and activation properties of novel ligands. Therefore, we focused on five ester odorants (Figure 2a) that are structurally related to amylbutyrate. After superposition of the ligands of interest over amylbutyrate in the vertical binding mode (for details see Supporting Information, Section 5a), binding to the hydrophilic belt of hOR2AG1 (Ser263\(^{51}\), Ser264\(^{52}\), and Thr279\(^{42}\)) was assessed in 10 ns of free MD simulations (Supporting Information, Table S1). Comparison of simulations of the full ligand set in the WT protein with 10 ns and 100 ns trajectory length (Supporting Information, Table S1) showed that 10 ns of simulation is sufficient for sampling the contacts between protein and ligand in our model (for details see Supporting Information, Section 5b). We investigated whether the ligands can form temporary hydrogen bonds to Ser263\(^{51}\) and Ser264\(^{52}\) and robust hydrogen bonds to Thr279\(^{42}\) during simulations in at least one of the employed orientations. While phenylethylacetate and phenirate fulfilled our activity criterion, prenylacetate, isoamylbenzoate, and isopentylacetate failed to do so (Figure 2a).

The simulations were then compared to \(\text{Ca}^{2+}\) imaging measurements analyzing relative changes in hOR2AG1 activation potencies of tested odorants compared to amylbutyrate. In agreement with the simulations, phenylethylacetate and phenirate were experimentally found to be as active as amylbutyrate, whereas prenylacetate and isoamylbenzoate were significantly less active. Isopentylacetate had a reduced activity in comparison to amylbutyrate, though a t-test showed no significance for this result (Figure 2b). Other substances that were tested experimentally for hOR2AG1 activation are listed in the Supporting Information, Figure S6. Thus, MD simulations predicted the activity of five novel odorants qualitatively correctly. Earlier receptor-based approaches for the analysis of receptor/odorant pairs employed docking methods\(^{[11–21]}\) and could therefore only predict if a ligand sterically fits into a binding cavity. In our study, all six investigated odorants fit into the cavity, but only three out of six could fully activate the receptor. This was exactly predicted by our dynamic homology modeling approach, as it takes the dynamic interplay between ligand and receptor into account.

Analyzing our results, we propose a ligand selectivity filter for the recognition of a minimal distinct local molecular shape, a so-called odotope,\(^{[10]}\) in hOR2AG1. The binding niche contains two hydrophobic cavities connected via a belt.
of hydrophilic residues (Ser263, Ser264, Thr279, Figure 1a and Figure 3a). Ala104 is in van der Waals contact with the cytoplasmic hydrophobic cavity, which is large enough to incorporate a methyl to propyl group. The hydrophilic belt itself is selective for the recognition of an ester moiety. Phe206 and Val269 form a size selective filter close to the hydrophilic belt, so that next to the ester moiety of the ligand, only unbranched methylene groups can exist. Larger residues, for example, a phenyl group, form repulsive van der Waals interactions with Phe206. This filter may allow the receptor to be activated by multiple compounds, as long as they exhibit the R-CH₂-COO-CH₂-R’ odotope. There seems to be a maximal side-chain size, as compounds such as alusus, hexylacetate, and allylethanoate, which all contain the odotope, cannot activate the receptor (compare Supporting Information, Figure S7a), as is the case for phenylethylacetate. This filter may allow the receptor to be activated by multiple compounds, as long as they exhibit the R-CH₂-COO-CH₂-R’ odotope. Phenirate is a exception, because its binding mode differs from amylobutyrate by forming hydrogen bonds with the hydrophilic belt through ester and ether moieties in parallel (Supporting Information, Figure S7b). Possibly, this ability to bind in a different binding mode may explain why for this ligand, an isopropyl group next to the ester moiety is tolerated.

Ligand-based methods showed a good performance to discriminate between activating and inactive ligands for other olfactory receptors, and could even elucidate the structure of new odorants. To cross-check if the six ligands used in this study could reveal their activation potency by such an approach, we created pharmacophore models for the ligands with the help of the PharmaGist webservice. The derived pharmacore model could identify all investigated ligands as putative binders for hOR2AG1, but could not distinguish between active and inactive compounds (see Supporting Information, Section 5c, Figure S12 and Table S3), either due to the small size of our employed ligand set, or because hOR2AG1 can distinguish between very small differences in the ligand scaffold. We therefore continue our investigation with our receptor-based “selectivity filter” model.

A very sensitive test for the selectivity filter is the introduction of a mutation, which can change the selectivity filter in a predictable way. Isoamylbenzoate, which was found to be inactive in experimental analysis (Figure 2b), cannot bind the hydrophilic belt of WT hOR2AG1 owing to steric hindrance by Phe206. However, mutation to valine results in the required hydrogen bonding to all three hydrophilic residues in simulations of isoamylbenzoate in the F206V mutant (Figure 3b and Supporting Information, Table S1.). In agreement with this expectation, the F206V mutant showed a significant increase in receptor activation by isoamylbenzoate compared to the WT receptor (Figure 3c), whereas the amount of expressed protein remained unchanged (Supporting Information, Figure S8c). Thus, we can selectively alter receptor function based on computational information on the proposed binding niche by site-specific point mutation.

We extended our results with hOR2AG1 to predict ligands for orphan ORs. If the identified constellation of ligand-binding residues generally plays a role in ester recognition, ORs with the same amino acids in corresponding positions should also be able to be activated by amylobutyrate. We tested this hypothesis by modeling and functionally characterizing hOR2AG2 and mOR283-2, and could identify amylobutyrate as ligand for both ORs (Supporting Information, Figure S9 and Section 5d).

In conclusion, by combining dynamic homology modeling with site-directed mutagenesis and functional analysis, we provided a molecular model of the ligand-binding niche of hOR2AG1 within a receptor model. We could deduce a quantitative criterion for receptor activation by ligands based on computed hydrogen-bond contact frequencies to amino acids forming the ligand binding site. This information on the ligand selectivity filter in hOR2AG1 helped us to get insight into detection and discrimination of volatile, highly hydrophobic, and flexible ligands by olfactory receptors. Thereby, we were able to predict the activation capability of novel odorants. The dynamic model correctly predicts alterations in receptor function upon mutation for activation by ligands that do not activate the WT protein. Dynamic homology modeling may be applied in the future for deorphanization of ORs and to provide a valid basis for OR-based drug design.

Received: June 10, 2011
Revised: October 10, 2011
Published online: December 5, 2011

Keywords: fragrances · functional characterization · molecular dynamics · molecular modeling · receptors