

## **Title**

A Review of the Human Sigma-1 Receptor Structure

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## **Abstract**

The Sigma-1 Receptor (S1R) is a small, ligand-regulated integral membrane protein involved in cell homeostasis and the cellular stress response. The receptor has a multitude of protein and small molecule interaction partners with therapeutic potential. Newly reported structures of the human S1R in ligand-bound states provides essential insights into small molecule binding in the context of the overall protein structure. The structure also raises many interesting questions and provides an excellent starting point for understanding the molecular tricks employed by this small membrane receptor to modulate a large number of signaling events. Here, we review insights from the structures of ligand-bound S1R in the context of previous biochemical studies and propose, from a structural viewpoint, a set of important future directions.

## Introduction

The Sigma-1 Receptor (S1R) presents an extremely compelling cell-biological and structural puzzle. Despite its small size S1R acts to regulate the activity of a large number of cellular proteins and is itself regulated by small molecule binding <sup>1</sup>. The reported protein interaction partners include ion channels (both ligand and voltage gated), GPCRs, transcription factors and the endoplasmic reticulum (ER) chaperone protein BiP <sup>2</sup>. Among the large number of small molecules that S1R binds to are cocaine <sup>3, 4</sup> (a stimulant and drug of abuse), haloperidol <sup>5</sup> (an antipsychotic), fluvoxamine <sup>6</sup> (an antidepressant), steroid hormones such as progesterone <sup>7</sup>, and single-chain lipid-like compounds such as sphingosine <sup>8</sup> and myristic acid <sup>9</sup>. Although S1R is thought to reside primarily in the mitochondria associated membrane (MAM) of the ER <sup>2</sup>, it has been reported to relocate to the plasma membrane and the nuclear membrane <sup>10-13</sup>. The physiological processes that S1R is involved in include neurotransmission, regulation of intracellular calcium concentrations, and cell survival. S1R dysfunction has been implicated in neurological disorders such as depression and addiction <sup>14</sup>, and neuropathic pain <sup>15</sup>.

## Overview of S1R sequence and structure

The existence of S1R has been known from pharmacological and radio-ligand binding studies for ~40 years <sup>5, 16</sup>, although the protein itself was not discovered for another twenty years <sup>17</sup>. Based on its amino acid sequence, S1R is a member of the *ERG2* family of membrane proteins, having approximately 33% identity and 66% similarity to the fungal  $\Delta 8 \rightarrow \Delta 7$  sterol isomerases <sup>17, 18</sup> (**Figure 1**). Now, another twenty years after the identification of the amino acid sequence, the first structure of full-length human S1R has been reported <sup>19</sup>. Two trimeric structures of the receptor bound to different small molecule ligands were determined (**Figure 2A**). One ligand, PD144418, is a known antagonist.

The S1R subunit structure has at its center two anti-parallel beta-sheets that form a squashed barrel-like structure (**Figure 2B**). The arrangement of the beta-sheets places it within the cupin fold family, also known as the jelly-roll family. The cupin fold is functionally versatile with members including metalloenzymes from several enzyme classes as well as seed storage proteins <sup>20</sup>. Metalloenzymes with the cupin fold have been observed to use Fe, Mn, Ni, Zn, Co, and Cu <sup>20</sup>, and include the oxidoreductases thiol dioxygenase <sup>21</sup> and 2-oxoglutarate oxygenase <sup>22</sup>, the lyase ectoine synthase, and the hydrolase KdgF <sup>23</sup>. The yeast sterol isomerases that are most similar in sequence to S1R (see below) have a modest affinity

to  $\text{Zn}^{+2}$ , but S1R does not bind metals with high affinity <sup>24</sup>, nor does it exhibit isomerase activity <sup>17</sup>.

The strands of the two sheets in the cupin fold of S1R are rotated at a  $\sim 30\text{-}40^\circ$  angle to each other. The two sheets of S1R make few noncovalent contacts, with the most conspicuous being a hydrogen bond between the backbone amide of Met90 in the highly curved strand 2 of the larger sheet to the sidechain hydroxyl of Ser113 in strand 4 in the smaller sheet. In the *ERG2* family, only serine or threonine are found at position 113 suggesting that this may be an important contact for structural stability (**Figure 1**).

The  $\beta$ -sheets of S1R are flanked on both the N- and C-termini by helical regions (**Figure 2BC**). The N-terminus contains the transmembrane helix (residues  $\sim 8\text{-}32$ ), which is followed by two helices (denoted A and B) that form a helical hairpin that lies on the surface of the larger 6-stranded sheet. The C-terminal region contains two helices (denoted D and E), which form a flat, hydrophobic surface that likely interacts with membrane <sup>19</sup>. In solution nuclear magnetic resonance (NMR) studies of an N-terminally truncated S1R construct the residues  $\sim 183\text{-}189$  and  $197\text{-}204$  were shown to interact with detergent acyl chains <sup>25</sup>. These residues are juxtaposed in the receptor and are probably strongly associated with the membrane (**Figure 2D**).

## **The S1R ligand binding pocket and pharmacophore**

In the receptor structures the ligands bind at a similar position within the  $\beta$ -sheets, and the binding site is analogous to the active site of the cupin fold metalloenzymes. Ligands bind to protein with a 1:1 stoichiometry and each ligand contacts only a single subunit within the trimer. The ligands bind in extended conformations and are surrounded by protein on all sides. The binding site is largely consistent with earlier sequence analysis: because S1R ligands are able to bind to the *ERG2* proteins in yeast and inhibit sterol isomerase activity, it was predicted that the two regions of highest amino acid conservation between S1R and the *ERG2* would be involved in ligand binding <sup>26</sup>. These regions were later named the steroid binding domain-like (SBDL) 1 (residues 91-109) and 2 (residues 176-194) <sup>27</sup> (**Figure 1**), and the importance of these regions in ligand binding was supported by derivatization studies using photoreactive analogs of cocaine <sup>27</sup> and fenpropimorph <sup>28</sup>. Chemical crosslinking and radio-ligand transfer between amino acids in SBDL1 and SBDL2 elegantly showed that the two regions are positioned close to each other in the folded receptor <sup>28, 29</sup>. In a satisfying confirmation of those predictions, the S1R structures show that SBDL1 and SBDL2 envelope

the bound ligands (**Figure 3A**) and account for the majority of the residues responsible for the primary hydrophobic site of the ligand pharmacophore (see below). SBDL1 consists of a  $\beta$ -hairpin (strands 2 and 3) within the larger of the two sheets and SBDL2 is a helix (helix D) that lies on top of the ligand binding site and also forms part of the membrane attachment region along with helix E.

Before the structure of S1R was known, a large number of studies reporting mutations, deletions, and labelling with photoreactive probes were reported (**Table 1**). The results of mutational studies are strongly supportive of the structural model, with most of the substitutions having large effects on ligand affinity found close to the ligand binding site (**Figure 3B**). The ligand pharmacophore includes a positive ionizable feature, which is frequently a basic amine. This can now be seen in the structures to interact directly with E172 in the binding pocket. In turn, E172 is stabilized by an interaction with D126 and Y103. A role for residues E172 and D126 in ligand binding was discovered by Seth et al.<sup>11</sup>, who tested glycine substitutions for each acidic residue in the C-terminal half of S1R for ligand binding. Substitution for either D126 or E172 abrogated drug binding<sup>11</sup>, and later studies suggested that E172 was especially important, as even a conservative substitution for aspartic acid at this position abolished ligand binding<sup>30</sup>. The importance of the hydroxyl of Y103 was tested with a phenylalanine substitution in an early study of ligand binding mutants and resulted in reduced affinity for the agonist (+)-pentazocine and the antagonist NE-100<sup>31</sup>.

Some substitutions are further from the binding site but have large effects on affinity. Most notably this includes R119A<sup>30</sup>, which is in a loop connecting strands 4 and 5, with the sidechain pointing away from the binding pocket. Elevated crystallographic B-factors for this residue indicate that it may be relatively mobile. However, the basic sidechain of R119 is at the oligomeric interface and along with His116 makes an intermolecular salt bridge to D195 of an adjacent subunit. R119 also makes an intermolecular hydrogen bond to T198. Thus, the R119A substitution may destabilize oligomerization, which has been correlated with ligand binding<sup>32</sup>. In that study, substitutions at G87 and G88 within SBDL1 also resulted in decreased ligand binding and decreased oligomer stability. G87 and G88 can now be seen to form a Type I' turn in the  $\beta$ -hairpin turn of the SBDL1. Position 88 is then required to be a glycine for the stability of the turn and is strictly conserved (**Figure 1**). However, the  $\beta$ -hairpin turn is found also at the 3-fold symmetry axis of the trimer and thus disruption of the turn is likely to cause also disassembly of the oligomer. Position 87 could, in theory, tolerate

non-glycine amino acids, but the larger sidechain would likely clash with the adjacent subunit.

C-terminal deletions on ligand binding can also be understood in terms of the structure. Deleting five or seven C-terminal residues, which has small or modest effects on ligand binding, removes approximately a single turn of helix E that has no long-range contacts. By contrast, deleting fourteen C-terminal residues would be expected to remove several long-range contacts that helix E makes with SBDL1 and the loop between helices B and C. Indeed, this deletion results in little or no ligand binding<sup>27, 30</sup>.

Additional amino acids have been suggested to be in direct contact with ligand because they are derivatised by photoreactive probes based on S1R ligands. This includes C94<sup>29</sup>, H154<sup>33</sup>, and D188<sup>27</sup> (**Figure 3B**). H154 is in contact with ligand at the narrow end of the cupin barrel, whereas C94 is at the other end of the barrel in strand 2. Although C94 is in a strand within SBDL1, the reactive thiol sidechain points away from the binding pocket, suggesting that either flexibility in the strand or mobility of the ligand within the binding site allows derivatization, or that the sidechain lies along the binding pathway. Crystallographic B-factors are increased for both complexes in ligand atoms that are nearer the ‘open’ end of the cupin barrel suggesting that bound ligands may have increased degrees of freedom here. D188 is at the end of helix D and within SBDL2, but like C94, it is at the more open end of the cupin barrel and while there are no direct interactions with bound ligand, it can be envisioned how flexibility in the protein or ligand might facilitate derivatisation.

S1R binds a chemically diverse range of small molecules, which has motivated development of S1R ligand pharmacophore models that can now be understood in the context of the receptor structure (**Figure 4**). The pharmacophore models have been developed from analyses of known S1R ligands as well as binding affinity measurements of systematically substituted panels of ligands<sup>34-50</sup>. The first pharmacophore models developed after separation of the S1R binding site from the Sigma 2 Receptor binding site<sup>51, 52</sup> were based on disubstituted piperidines<sup>36</sup> and N-substituted phenylalkylamines<sup>37</sup>. Subsequent models have been largely consistent with these models in that a central basic amine nitrogen atom, or more generally a positive ionizable feature, is flanked on either side, in a more or less linear arrangement by a set of hydrophobic features (**Figure 4A**). On one side of the nitrogen is a primary hydrophobic feature centered 6-10 Å from the nitrogen atom, and on the other side is a secondary hydrophobic feature that is centered 2.5-3.9 Å from the nitrogen atom, with the latter being tolerant of bulky substituents with little change in affinity<sup>37</sup>.

PD144418 or 4-IBP fit well to the S1R pharmacophore, and as noted above the key amino acid stabilizing the positive ionizable feature is E172, with D126 and Y103 playing a supporting role (**Figure 4B**). Although some degree of ambiguity in ligand orientation can arise in fitting of the ligands to the experimentally derived electron density, the binding site for the larger, primary hydrophobic feature appears to be at the membrane proximal and more open end of the cupin barrel, while the secondary hydrophobic feature is within the narrower end of the cupin  $\beta$ -barrel that is further from the membrane. Further support for this orientation comes from the photoreactive probes of the binding pocket: C94 and D188 at the open end of the cupin barrel react with the photoprobes [ $^{125}$ I]IABM <sup>29</sup> and [ $^{125}$ I]IACoc <sup>27</sup>, respectively, which have reactive groups on the primary hydrophobic feature, whereas H154 at the smaller end of the cupin barrel reacts with the photoprobe 4-NPPC12 <sup>33</sup>, which has a reactive nitrophenol on the secondary hydrophobic feature.

Several research groups have expanded on the earlier S1R pharmacophore model, with some variation between models expected depending on the panel of ligands used to develop the models. Laggner et al., for example, split the primary hydrophobic group into two hydrophobic groups and inserted a third hydrophobic group between it and the central nitrogen <sup>43</sup> (**Figure 4C**). In the model developed by Zampieri et al., the primary hydrophobic group is divided into a hydrophobic aromatic closer to the central positive ionizable feature and another hydrophobic site further out <sup>46</sup> (**Figure 4D**). The Zampieri model includes also a hydrogen bond acceptor site, and from the S1R structures the sidechains of residues Y103 and T181 are close enough to be potential hydrogen bond donors. Substitutions at Y103 are known to affect drug binding <sup>30, 31</sup>, however Y103 interacts with the critical residue E172 and also ring stacks with the ligand at the membrane-proximal end of the binding pocket. A T181A substitution does not affect (+)-pentazocine binding <sup>30</sup>, however that ligand does not appear to have the expected hydrogen bond acceptor.

## Structural implications for membrane topology

Sequence-based transmembrane helix predictors had reliably predicted for S1R an N-terminal transmembrane helix at residues ~9-30, which was confirmed by the structure. The transmembrane helix may also contain a signal peptide directing it to the ER membrane <sup>53</sup>, and homologs of S1R and the fungal sterol isomerases can be found in  $\gamma$ - and  $\delta$ -proteobacteria that are 35% to 38% identical in sequence (**Figure 1**) but lack the first transmembrane helix. A second transmembrane helix was expected based on analysis of

amino acid hydrophobicity <sup>10</sup> and predictors of transmembrane helices <sup>54</sup>. Studies of the S1R membrane topology indicated that the N- and C-termini of S1R reside on the same side of the membrane, consistent with an even number of transmembrane domains <sup>2, 10, 55</sup>. In addition, solution NMR studies of a truncated form of S1R reconstituted into a mixture of detergent and lipids were consistent with a helical conformation of the SBDL1 and assigned to a second transmembrane domain <sup>54</sup>. The structure of the intact receptor however, indicates only a single transmembrane helix at the N-terminus, and that the SBDL1 adopts a  $\beta$ -hairpin conformation <sup>19</sup>.

Notably, membrane topology studies of S1R have been ambiguous: in oocyte studies both termini were determined to be in the cytosol <sup>10</sup>, whereas in CHO cells <sup>2</sup> or human embryonal kidney cells <sup>55</sup> both termini were found to be in the ER lumen or on the extracellular side of the plasma membrane, respectively. The surprising single transmembrane domain architecture of S1R places the vast majority of the protein on one side of the membrane and leaves only a handful of poorly conserved residues on the other side. Thus, the S1R membrane topology makes it difficult to rationalize how the receptor could interact with proteins both in the cytoplasm and in the ER lumen. Interactions with several proteins or regions of protein that are found exclusively in the cytoplasm or the nucleus, including the C-terminus of the GluR1 subunit of the ionotropic glutamate receptor <sup>57</sup>, STIM1 <sup>58</sup>, and emerin <sup>12</sup>, are consistent with S1R being a Type I membrane protein with a cytosolic C-terminus. However, an interaction at the C-terminus of S1R with BiP has been shown in cell extracts <sup>2</sup> and *in vitro* <sup>25</sup>. Although BiP can relocate to the cytoplasm at low concentrations <sup>56</sup>, it is predominantly found in the ER lumen. Thus, further work is necessary to understand the biological relevance of this interaction.

## Oligomerization

Early evidence for the formation of S1R oligomers came from studies in which selective photoreactive-probes reacted with higher molecular weight species <sup>28</sup>. More recently, it has been shown that receptor expressed and purified from either *E. coli* <sup>32</sup> or insect cells <sup>19</sup> forms a mixture of oligomeric forms and that ligands stabilize the oligomeric forms of the protein <sup>32</sup>. The existence of oligomeric forms of S1R in membranes has been shown also in COS-7 cells by FRET after co-transfection with S1R-GFP2 and S1R-YFP <sup>59</sup>, and, consistent with the *in vitro* studies <sup>32</sup>, the introduction of ligands (specifically antagonists) stimulated the formation of higher order species.

The structures of ligand-bound S1R observed by X-ray crystallography are homotrimeric, which was surprising in light of previous studies suggesting that an even-number of subunits was likely. For example, only ~50% of S1R preparations were observed to bind (+)-pentazocine<sup>60</sup>, and the photoreactive-probe 4-NCCP12 derivatizes no more than 50% of the receptor molecules<sup>33</sup>. The proteins in these binding studies were recombinantly expressed and purified from *E. coli*, and it remains possible that on average about half of the proteins are fully folded under those conditions. However, a similar level of derivatization by 4-NCCP12 was seen for S1R expressed in COS-7 cells and guinea pig liver microsomes. In addition, analytical size exclusion chromatography results from Gromek et al.<sup>32</sup>, were most consistent with a tetramer and a larger species consisting of approximately 6 to 8 subunits, whereas cellular fluorescence data was consistent with mostly monomeric and dimeric forms of the receptor<sup>59</sup>. The role of S1R oligomerization, including that of the alternative oligomeric states, remains unknown.

In the S1R trimer structure determined by X-ray crystallography, extensive polar and nonpolar inter-subunit contacts are made over a large surface area<sup>19</sup>. Some contacts, including a hydrophobic cluster formed by the three phenylalanines at position 191, are found along the 3-fold symmetry axis. Other notable contacts are made toward the periphery of the trimer in which residues from the loop connecting strands 6 and 7 interact with residues in an adjacent subunit. W136, at the end of strand 6, is at the center of a hydrophobic core of residues from an adjacent subunit. Packing on top and at the side of W136 are F83, M90, and A92, and making contacts below the tryptophan are T109, A110, and L111. These residues are from strands 1 and 2, and the loop between strands 3 and 4, respectively, of the adjacent subunit. Intriguingly, W136 and T141 are within the stretch of residues ~135-165 that are predicted from amino acid sequence to have a high degree of intrinsic disorder. This suggests that despite a large number of inter-subunit contacts, the stabilizing effects of these enthalpic contacts may be offset by entropic costs from restricting flexibility in these residues.

Other inter-subunit contacts include polar interactions between the sidechains of T141 of strand 6 and the sidechains of H54 and E55 in helix B, and a bifurcated hydrogen bond between the sidechain of Q194 and the peptide bond joining A183 and F184. Many of the sidechains forming inter-subunit contacts, including those of H54, E55, W136, T141, F191 and Q194, are not highly conserved in the fungal sterol isomerases suggesting that this observed mode of oligomerization may be unique to the S1Rs.



## Future directions

The ligand-bound structures of S1R<sup>19</sup> have provided an enormous leap forward in the study of S1R and have helped to unify and extend several decades of biochemical studies. Yet, numerous structural questions remain. In this section, we highlight some of the most interesting unanswered questions in light of the receptor structure.

*What are the conformational changes associated with receptor activation?* The receptor structures are bound to two different S1R ligands, one of which is a known antagonist and the other may be an agonist. Nonetheless, the two protein structures are very similar (0.4 Å all atom RMSD)<sup>19</sup>. Thus, the mechanism of receptor activation remains a mystery. Also unknown is whether the receptor adopts multiple conformations with different activities or only exists in an 'on' or an 'off' state. Ultimately the structures of S1R in the apo state and the agonist bound state will be needed to provide insights into the conformational changes associated with receptor activity. Further studies of changes in local and global flexibility in the receptor associated with ligand binding is likely to be necessary to fully understand receptor activation<sup>61</sup>.

*Where is the interaction site for protein-protein interactions?* Identification of the structural elements and amino acids involved in the large number of reported protein-protein interactions (reviewed in<sup>1</sup>) is required to understand the specificity of S1R signaling. It is possible that the interface for protein-protein interactions is only accessible in the monomeric form<sup>32, 62, 63</sup>, however, the surface-exposed helices A and B are attractive potential sites of interaction based on the trimeric structures. Residues 61-65 in helix B form a putative SUMO interaction site, and peptide interference assays are consistent with the binding of the GluR1 cytoplasmic tail near this region<sup>57</sup>. Furthermore, the helices lie against the large β-sheet enclosing the ligand binding site, and helix B contacts L106, which has been proposed to be involved in discriminating between agonist and antagonist<sup>31</sup>. Thus, one can begin to hypothesize about potential allosteric pathways between the ligand and protein binding sites. The prospects for investigating such hypotheses are good: several protein-protein interactions of S1R have been directly observed<sup>55, 62-65</sup>, and advances in recombinant expression and purification of the receptor<sup>19, 54, 60, 66</sup> make it likely that biophysical assays will soon complement *in vivo* studies of protein-protein interactions. Such studies may also help to reconcile contradictory information on the S1R membrane topology.

*How is the S1R ligand binding site able to accommodate chemically diverse ligands?* Despite being bound to different ligands, the S1R ligand binding site architecture is essentially unchanged in the two crystallographic structures. Thus, to understand the physical

properties of the S1R binding pocket additional structures of S1R bound to other ligands are needed, and likely also information on dynamics of both the protein and the bound drug. Especially informative may be structural information on receptor complexes with neurosteroids and single-chain lipid-like compounds, since they lack positive ionizable features. With regard to the binding of diverse ligands, the  $\beta$ -sheet structure of the S1R binding pocket may be expected to provide the flexibility needed for adaptation of the binding site <sup>67</sup>. A useful comparator is the fatty-acid binding protein family, since they are able to bind both long-chain fatty acids and bile salts, and have also a central binding pocket buried between antiparallel  $\beta$ -sheets <sup>68</sup>.  $\beta$ -sheets also facilitate large-scale correlated motions <sup>69</sup>, which may be important for allosteric signaling outward from the buried ligand binding pocket.

*Does oligomerisation regulate S1R interactions?* Based on previous work the trimeric structure of S1R was unexpected. Available data suggest that this is not a function of the protein expression system since protein prepared similarly to that which was crystallized also displays polydisperse oligomerisation <sup>19</sup>. Thus, the consequences of oligomerisation for receptor function, including downstream protein-protein interactions, need to be better understood.

*How does drug get in and out of the binding pocket?* The central fold of S1R is  $\beta$ -barrel-like, having a narrower, essentially closed, end where the two sheets are closely apposed, and a more open end in which the longer strands of the large sheet curve toward the smaller sheet. However the open end of the central  $\beta$ -barrel is capped by helices D and E and there is no obvious entry or exit pathway to the ligand binding site. Being related to the sterol isomerases would suggest access occurs through the region closest to the membrane, which would be also consistent with the presumed binding pathway for enzymes of the cupin fold family. However, the  $\beta$ -strands at the narrow end of the barrel have elevated crystallographic B-factors and are predicted from sequence to be flexible, thus dynamics here may provide a transient pathway from solvent to the ligand binding site.

*What is the molecular basis of S1R chaperone activity?* No further studies on the chaperone activity have appeared since Hayashi and Su first identified such activity and linked it to the C-terminal half of S1R <sup>2</sup>. Important questions remain, such as whether the full-length receptor exhibits similar activity and whether the chaperone activity is ligand-dependent. Also important is to test the relatedness of the S1R chaperone domain function to known families of protein chaperones <sup>70</sup>.

Many of the questions outlined above have been elucidated previously, but the new S1R structures provide a firmer foundation from which these questions can be approached. Without a doubt S1R will continue to throw up surprises as the answers to these questions become more clear.

## References

- [1] Su, T. P., Su, T. C., Nakamura, Y., and Tsai, S. Y. (2016) The Sigma-1 Receptor as a Pluripotent Modulator in Living Systems, *Trends Pharmacol Sci* 37, 262-278.
- [2] Hayashi, T., and Su, T. P. (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival, *Cell* 131, 596-610.
- [3] Sharkey, J., Glen, K. A., Wolfe, S., and Kuhar, M. J. (1988) Cocaine binding at sigma receptors, *Eur J Pharmacol* 149, 171-174.
- [4] Kahoun, J. R., and Ruoho, A. E. (1992) (125I)iodoazidococaine, a photoaffinity label for the haloperidol-sensitive sigma receptor, *Proc Natl Acad Sci U S A* 89, 1393-1397.
- [5] Su, T. P. (1982) Evidence for sigma opioid receptor: binding of [3H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain, *J Pharmacol Exp Ther* 223, 284-290.
- [6] Narita, N., Hashimoto, K., Tomitaka, S., and Minabe, Y. (1996) Interactions of selective serotonin reuptake inhibitors with subtypes of sigma receptors in rat brain, *Eur J Pharmacol* 307, 117-119.
- [7] Su, T. P., London, E. D., and Jaffe, J. H. (1988) Steroid binding at sigma receptors suggests a link between endocrine, nervous, and immune systems, *Science* 240, 219-221.
- [8] Ramachandran, S., Chu, U. B., Mavlyutov, T. A., Pal, A., Pyne, S., and Ruoho, A. E. (2009) The sigma1 receptor interacts with N-alkyl amines and endogenous sphingolipids, *Eur J Pharmacol* 609, 19-26.
- [9] Tsai, S. Y., Pokrass, M. J., Klauer, N. R., Nohara, H., and Su, T. P. (2015) Sigma-1 receptor regulates Tau phosphorylation and axon extension by shaping p35 turnover via myristic acid, *Proc Natl Acad Sci U S A* 112, 6742-6747.
- [10] Aydar, E., Palmer, C. P., Klyachko, V. A., and Jackson, M. B. (2002) The sigma receptor as a ligand-regulated auxiliary potassium channel subunit, *Neuron* 34, 399-410.
- [11] Seth, P., Ganapathy, M. E., Conway, S. J., Bridges, C. D., Smith, S. B., Casellas, P., and Ganapathy, V. (2001) Expression pattern of the type 1 sigma receptor in the brain and identity of critical anionic amino acid residues in the ligand-binding domain of the receptor, *Biochim Biophys Acta* 1540, 59-67.
- [12] Tsai, S. Y., Chuang, J. Y., Tsai, M. S., Wang, X. F., Xi, Z. X., Hung, J. J., Chang, W. C., Bonci, A., and Su, T. P. (2015) Sigma-1 receptor mediates cocaine-induced transcriptional regulation by recruiting chromatin-remodeling factors at the nuclear envelope, *Proc Natl Acad Sci U S A* 112, E6562-6570.
- [13] Su, T. P., Hayashi, T., Maurice, T., Buch, S., and Ruoho, A. E. (2010) The sigma-1 receptor chaperone as an inter-organelle signaling modulator, *Trends Pharmacol Sci* 31, 557-566.
- [14] Kourrich, S., Su, T. P., Fujimoto, M., and Bonci, A. (2012) The sigma-1 receptor: roles in neuronal plasticity and disease, *Trends Neurosci* 35, 762-771.
- [15] Zamanillo, D., Romero, L., Merlos, M., and Vela, J. M. (2013) Sigma 1 receptor: a new therapeutic target for pain, *Eur J Pharmacol* 716, 78-93.

- [16] Martin, W. R., Eades, C. G., Thompson, J. A., Huppler, R. E., and Gilbert, P. E. (1976) The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog, *J Pharmacol Exp Ther* 197, 517-532.
- [17] Hanner, M., Moebius, F. F., Flandorfer, A., Knaus, H. G., Striessnig, J., Kempner, E., and Glossmann, H. (1996) Purification, molecular cloning, and expression of the mammalian sigma1-binding site, *Proc Natl Acad Sci U S A* 93, 8072-8077.
- [18] Moebius, F. F., Reiter, R. J., Hanner, M., and Glossmann, H. (1997) High affinity of sigma 1-binding sites for sterol isomerization inhibitors: evidence for a pharmacological relationship with the yeast sterol C8-C7 isomerase, *Br J Pharmacol* 121, 1-6.
- [19] Schmidt, H. R., Zheng, S., Gurpinar, E., Koehl, A., Manglik, A., and Kruse, A. C. (2016) Crystal structure of the human sigma receptor, *Nature* 532, 527-530.
- [20] Dunwell, J. M., Purvis, A., and Khuri, S. (2004) Cupins: the most functionally diverse protein superfamily?, *Phytochemistry* 65, 7-17.
- [21] Stipanuk, M. H., Simmons, C. R., Karplus, P. A., and Dominy, J. E., Jr. (2011) Thiol dioxygenases: unique families of cupin proteins, *Amino Acids* 41, 91-102.
- [22] Aik, W., McDonough, M. A., Thalhammer, A., Chowdhury, R., and Schofield, C. J. (2012) Role of the jelly-roll fold in substrate binding by 2-oxoglutarate oxygenases, *Curr Opin Struct Biol* 22, 691-700.
- [23] Hobbs, J. K., Lee, S. M., Robb, M., Hof, F., Barr, C., Abe, K. T., Hehemann, J. H., McLean, R., Abbott, D. W., and Boraston, A. B. (2016) KdgF, the missing link in the microbial metabolism of uronate sugars from pectin and alginate, *Proc Natl Acad Sci U S A* 113, 6188-6193.
- [24] Basile, A. S., Paul, I. A., Mirchevich, A., Kuijpers, G., and De Costa, B. (1992) Modulation of (+)-[3H]pentazocine binding to guinea pig cerebellum by divalent cations, *Mol Pharmacol* 42, 882-889.
- [25] Ortega-Roldan, J. L., Ossa, F., and Schnell, J. R. (2013) Characterization of the Human Sigma-1 Receptor Chaperone Domain Structure and Binding Immunoglobulin Protein (BiP) Interactions, *J Biol Chem* 288, 21448-21457.
- [26] Moebius, F. F., Bermoser, K., Reiter, R. J., Hanner, M., and Glossmann, H. (1996) Yeast sterol C8-C7 isomerase: identification and characterization of a high-affinity binding site for enzyme inhibitors, *Biochemistry* 35, 16871-16878.
- [27] Chen, Y., Hajipour, A. R., Sievert, M. K., Arbabian, M., and Ruoho, A. E. (2007) Characterization of the cocaine binding site on the sigma-1 receptor, *Biochemistry* 46, 3532-3542.
- [28] Pal, A., Hajipour, A. R., Fontanilla, D., Ramachandran, S., Chu, U. B., Mavlyutov, T., and Ruoho, A. E. (2007) Identification of regions of the sigma-1 receptor ligand binding site using a novel photoprobe, *Mol Pharmacol* 72, 921-933.
- [29] Pal, A., Chu, U. B., Ramachandran, S., Grawoig, D., Guo, L. W., Hajipour, A. R., and Ruoho, A. E. (2008) Juxtaposition of the steroid binding domain-like I and II regions constitutes a ligand binding site in the sigma-1 receptor, *J Biol Chem* 283, 19646-19656.
- [30] Brune, S., Schepmann, D., Klempnauer, K. H., Marson, D., Dal Col, V., Laurini, E., Fermeglia, M., Wunsch, B., and Pricl, S. (2014) The sigma enigma: in vitro/in silico site-directed mutagenesis studies unveil sigma1 receptor ligand binding, *Biochemistry* 53, 2993-3003.

- [31] Yamamoto, H., Miura, R., Yamamoto, T., Shinohara, K., Watanabe, M., Okuyama, S., Nakazato, A., and Nukada, T. (1999) Amino acid residues in the transmembrane domain of the type 1 sigma receptor critical for ligand binding, *FEBS Lett* 445, 19-22.
- [32] Gromek, K. A., Suchy, F. P., Meddaugh, H. R., Wrobel, R. L., LaPointe, L. M., Chu, U. B., Primm, J. G., Ruoho, A. E., Senes, A., and Fox, B. G. (2014) The oligomeric states of the purified sigma-1 receptor are stabilized by ligands, *J Biol Chem* 289, 20333-20344.
- [33] Chu, U. B., Ramachandran, S., Hajipour, A. R., and Ruoho, A. E. (2013) Photoaffinity labeling of the sigma-1 receptor with N-[3-(4-nitrophenyl)propyl]-N-dodecylamine: evidence of receptor dimers, *Biochemistry* 52, 859-868.
- [34] Largent, B. L., Wikstrom, H., Gundlach, A. L., and Snyder, S. H. (1987) Structural determinants of sigma receptor affinity, *Mol Pharmacol* 32, 772-784.
- [35] Manallack, D. T., Wong, M. G., Costa, M., Andrews, P. R., and Beart, P. M. (1988) Receptor site topographies for phencyclidine-like and sigma drugs: predictions from quantitative conformational, electrostatic potential, and radioreceptor analyses, *Mol Pharmacol* 34, 863-879.
- [36] Gilligan, P. J., Cain, G. A., Christos, T. E., Cook, L., Drummond, S., Johnson, A. L., Kergaye, A. A., McElroy, J. F., Rohrbach, K. W., Schmidt, W. K., and et al. (1992) Novel piperidine sigma receptor ligands as potential antipsychotic drugs, *J Med Chem* 35, 4344-4361.
- [37] Glennon, R. A., Ablordeppey, S. Y., Ismaiel, A. M., el-Ashmawy, M. B., Fischer, J. B., and Howie, K. B. (1994) Structural features important for sigma 1 receptor binding, *J Med Chem* 37, 1214-1219.
- [38] Hudkins, R. L., Mailman, R. B., and DeHaven-Hudkins, D. L. (1994) Novel (4-phenylpiperidinyl)- and (4-phenylpiperazinyl)alkyl-spaced esters of 1-phenylcyclopentanecarboxylic acids as potent sigma-selective compounds, *J Med Chem* 37, 1964-1970.
- [39] Ucar, H., Cacciaguerra, S., Spampinato, S., Van derpoorten, K., Isa, M., Kanyonyo, M., and Poupaert, J. H. (1997) 2(3H)-benzoxazolone and 2(3H)-benzothiazolone derivatives: novel, potent and selective sigma1 receptor ligands, *Eur J Pharmacol* 335, 267-273.
- [40] Quaglia, W., Giannella, M., Piergentili, A., Pignini, M., Brasili, L., Di Toro, R., Rossetti, L., Spampinato, S., and Melchiorre, C. (1998) 1'-Benzyl-3,4-dihydrospiro[2H-1-benzothiopyran-2,4'-piperidine] (spipethiane), a potent and highly selective sigma1 ligand, *J Med Chem* 41, 1557-1560.
- [41] Gund, T. M., Floyd, J., and Jung, D. (2004) Molecular modeling of sigma 1 receptor ligands: a model of binding conformational and electrostatic considerations, *J Mol Graph Model* 22, 221-230.
- [42] Jung, D., Floyd, J., and Gund, T. M. (2004) A comparative molecular field analysis (CoMFA) study using semiempirical, density functional, ab initio methods and pharmacophore derivation using DISCOtech on sigma 1 ligands, *J Comput Chem* 25, 1385-1399.
- [43] Laggner, C., Schieferer, C., Fiechtner, B., Poles, G., Hoffmann, R. D., Glossmann, H., Langer, T., and Moebius, F. F. (2005) Discovery of high-affinity ligands of sigma1 receptor, ERG2, and emopamil binding protein by pharmacophore modeling and virtual screening, *J Med Chem* 48, 4754-4764.

- [44] Costantino, L., Gandolfi, F., Sorbi, C., Franchini, S., Prezzavento, O., Vittorio, F., Ronsisvalle, G., Leonardi, A., Poggesi, E., and Brasili, L. (2005) Synthesis and structure-activity relationships of 1-alkyl-4-benzylpiperidine and 1-alkyl-4-benzylpiperazine derivatives as potent sigma ligands, *J Med Chem* 48, 266-273.
- [45] Fontanilla, D., Johannessen, M., Hajipour, A. R., Cozzi, N. V., Jackson, M. B., and Ruoho, A. E. (2009) The hallucinogen N,N-dimethyltryptamine (DMT) is an endogenous sigma-1 receptor regulator, *Science* 323, 934-937.
- [46] Zampieri, D., Mamolo, M. G., Laurini, E., Florio, C., Zanette, C., Fermeglia, M., Posocco, P., Paneni, M. S., Pricl, S., and Vio, L. (2009) Synthesis, biological evaluation, and three-dimensional in silico pharmacophore model for sigma(1) receptor ligands based on a series of substituted benzo[d]oxazol-2(3H)-one derivatives, *J Med Chem* 52, 5380-5393.
- [47] Laurini, E., Da Col, V., Wunsch, B., and Pricl, S. (2013) Analysis of the molecular interactions of the potent analgesic S1RA with the sigma1 receptor, *Bioorg Med Chem Lett* 23, 2868-2871.
- [48] Diaz, J. L., Christmann, U., Fernandez, A., Luengo, M., Bordas, M., Enrech, R., Carro, M., Pascual, R., Burgueno, J., Merlos, M., Benet-Buchholz, J., Ceron-Bertran, J., Ramirez, J., Reinoso, R. F., Fernandez de Henestrosa, A. R., Vela, J. M., and Almansa, C. (2013) Synthesis and biological evaluation of a new series of hexahydro-2H-pyrano[3,2-c]quinolines as novel selective sigma1 receptor ligands, *J Med Chem* 56, 3656-3665.
- [49] Diaz, J. L., Christmann, U., Fernandez, A., Torrens, A., Port, A., Pascual, R., Alvarez, I., Burgueno, J., Monroy, X., Montero, A., Balada, A., Vela, J. M., and Almansa, C. (2015) Synthesis and structure-activity relationship study of a new series of selective sigma(1) receptor ligands for the treatment of pain: 4-aminotriazoles, *J Med Chem* 58, 2441-2451.
- [50] Diaz, J. L., Cuberes, R., Berrocal, J., Contijoch, M., Christmann, U., Fernandez, A., Port, A., Holenz, J., Buschmann, H., Laggner, C., Serafini, M. T., Burgueno, J., Zamanillo, D., Merlos, M., Vela, J. M., and Almansa, C. (2012) Synthesis and biological evaluation of the 1-arylpyrazole class of sigma(1) receptor antagonists: identification of 4-{2-[5-methyl-1-(naphthalen-2-yl)-1H-pyrazol-3-yloxy]ethyl}morpholine (S1RA, E-52862), *J Med Chem* 55, 8211-8224.
- [51] Hellewell, S. B., and Bowen, W. D. (1990) A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain, *Brain Res* 527, 244-253.
- [52] McCann, D. J., Weissman, A. D., and Su, T. P. (1994) Sigma-1 and sigma-2 sites in rat brain: comparison of regional, ontogenetic, and subcellular patterns, *Synapse* 17, 182-189.
- [53] Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions, *Nat Methods* 8, 785-786.
- [54] Ortega-Roldan, J. L., Ossa, F., Amin, N. T., and Schnell, J. R. (2015) Solution NMR studies reveal the location of the second transmembrane domain of the human sigma-1 receptor, *FEBS Lett* 589, 659-665.
- [55] Balasuriya, D., Stewart, A. P., and Edwardson, J. M. (2013) The sigma-1 receptor interacts directly with GluN1 but not GluN2A in the GluN1/GluN2A NMDA receptor, *J Neurosci* 33, 18219-18224.

- [56] Cha-Molstad, H., Sung, K. S., Hwang, J., Kim, K. A., Yu, J. E., Yoo, Y. D., Jang, J. M., Han, D. H., Molstad, M., Kim, J. G., Lee, Y. J., Zakrzewska, A., Kim, S. H., Kim, S. T., Kim, S. Y., Lee, H. G., Soung, N. K., Ahn, J. S., Ciechanover, A., Kim, B. Y., and Kwon, Y. T. (2015) Amino-terminal arginylation targets endoplasmic reticulum chaperone BiP for autophagy through p62 binding, *Nat Cell Biol* 17, 917-929.
- [57] Rodriguez-Munoz, M., Sanchez-Blazquez, P., Herrero-Labrador, R., Martinez-Murillo, R., Merlos, M., Vela, J. M., and Garzon, J. (2015) The sigma1 receptor engages the redox-regulated HINT1 protein to bring opioid analgesia under NMDA receptor negative control, *Antioxidants & redox signaling* 22, 799-818.
- [58] Srivats, S., Balasuriya, D., Pasche, M., Vistal, G., Taylor, C. W., Edwardson, J. M., and Murrell-Lagnado, R. D. (2015) The Sigma1 Receptor Competes with STIM1 to Bind Orai1 to Regulate Store Operated Calcium Entry (SOCE), *Biophysical Journal* 108, 128a-129a.
- [59] Mishra, A. K., Mavlyutov, T., Singh, D. R., Biener, G., Yang, J., Oliver, J. A., Ruoho, A., and Raicu, V. (2015) The sigma-1 receptors are present in monomeric and oligomeric forms in living cells in the presence and absence of ligands, *Biochem J* 466, 263-271.
- [60] Ramachandran, S., Lu, H., Prabhu, U., and Ruoho, A. E. (2007) Purification and characterization of the guinea pig sigma-1 receptor functionally expressed in Escherichia coli, *Protein Expr Purif* 51, 283-292.
- [61] Manglik, A., and Kobilka, B. (2014) The role of protein dynamics in GPCR function: insights from the beta2AR and rhodopsin, *Curr Opin Cell Biol* 27, 136-143.
- [62] Carnally, S. M., Johannessen, M., Henderson, R. M., Jackson, M. B., and Edwardson, J. M. (2010) Demonstration of a direct interaction between sigma-1 receptors and acid-sensing ion channels, *Biophys J* 98, 1182-1191.
- [63] Balasuriya, D., Stewart, A. P., Crottes, D., Borgese, F., Soriani, O., and Edwardson, J. M. (2012) The sigma-1 receptor binds to the Nav1.5 voltage-gated Na<sup>+</sup> channel with 4-fold symmetry, *J Biol Chem* 287, 37021-37029.
- [64] Crottes, D., Guizouarn, H., Martin, P., Borgese, F., and Soriani, O. (2013) The sigma-1 receptor: a regulator of cancer cell electrical plasticity?, *Frontiers in physiology* 4, 175.
- [65] Balasuriya, D., D'Sa, L., Talker, R., Dupuis, E., Maurin, F., Martin, P., Borgese, F., Soriani, O., and Edwardson, J. M. (2014) A direct interaction between the sigma-1 receptor and the hERG voltage-gated K<sup>+</sup> channel revealed by atomic force microscopy and homogeneous time-resolved fluorescence (HTRF(R)), *J Biol Chem*.
- [66] Gromek, K. A., Meddaugh, H. R., Wrobel, R. L., Suchy, F. P., Bingman, C. A., Primm, J. G., and Fox, B. G. (2013) Improved expression and purification of sigma 1 receptor fused to maltose binding protein by alteration of linker sequence, *Protein Expr Purif* 89, 203-209.
- [67] Emberly, E. G., Mukhopadhyay, R., Tang, C., and Wingreen, N. S. (2004) Flexibility of beta-sheets: principal component analysis of database protein structures, *Proteins* 55, 91-98.
- [68] Monaco, H. L. (2009) Review: the liver bile acid-binding proteins, *Biopolymers* 91, 1196-1202.
- [69] Fenwick, R. B., Orellana, L., Esteban-Martin, S., Orozco, M., and Salvatella, X. (2014) Correlated motions are a fundamental property of beta-sheets, *Nat Commun* 5, 4070.

- [70] Chu, U. B., and Ruoho, A. E. (2016) Biochemical Pharmacology of the Sigma-1 Receptor, *Mol Pharmacol* 89, 142-153.
- [71] Ganapathy, M. E., Prasad, P. D., Huang, W., Seth, P., Leibach, F. H., and Ganapathy, V. (1999) Molecular and ligand-binding characterization of the sigma-receptor in the Jurkat human T lymphocyte cell line, *J Pharmacol Exp Ther* 289, 251-260.
- [72] Palmer, C. P., Mahen, R., Schnell, E., Djamgoz, M. B., and Aydar, E. (2007) Sigma-1 receptors bind cholesterol and remodel lipid rafts in breast cancer cell lines, *Cancer Res* 67, 11166-11175.
- [73] Notredame, C., Higgins, D. G., and Heringa, J. (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment, *J Mol Biol* 302, 205-217.
- [74] Stivala, A., Wybrow, M., Wirth, A., Whisstock, J. C., and Stuckey, P. J. (2011) Automatic generation of protein structure cartoons with Pro-origami, *Bioinformatics* 27, 3315-3316.
- [75] Laskowski, R. A., and Swindells, M. B. (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery, *J Chem Inf Model* 51, 2778-2786.



SIR residues	Drug Tested <sup>a</sup>	Effect on binding	Reference
A13T/L28P/A86V	haloperidol	moderate	71
G87I	(+)-pentazocine	large	32
G87L	(+)-pentazocine	large	32
G88I	(+)-pentazocine	large	32
G88L	(+)-pentazocine	large	32
G91I	(+)-pentazocine	large	32
G91L	(+)-pentazocine	large	32
C94	[ <sup>125</sup> I]IABM	derivatized	29
C94A/V190C	(+)-pentazocine	no significant effect	29
H97A	(+)-pentazocine	moderate	32
S99A	(+)-pentazocine, NE-100	moderate	31
S101A	(+)-pentazocine	large	30
Y103A	(+)-pentazocine	large	30
Y103F	(+)-pentazocine, NE-100	large	31
L105A/L106A	(+)-pentazocine, NE-100	moderate	31
L105A/L106A/S99A	(+)-pentazocine	no significant effect	31
L105A/L106A/S99A	NE-100	large	31
F107A	(+)-pentazocine	large	30
R119A	(+)-pentazocine	large	30
Y120A	(+)-pentazocine	moderate	30
W121A	(+)-pentazocine	no significant effect	30
E123G	haloperidol	no significant effect	11
S125A	(+)-pentazocine	moderate	30
D126G	haloperidol	large	11
D126E	(+)-pentazocine	moderate	30
T127A	(+)-pentazocine	moderate	30
I128A	(+)-pentazocine	large	30
E138G	haloperidol	no significant effect	11
E144G	haloperidol	no significant effect	11
V145A	(+)-pentazocine	no significant effect	30
F146A	(+)-pentazocine	moderate	30
Y147A	(+)-pentazocine	no significant effect	30
E150G	haloperidol	no significant effect	11
H154	4-NPPC12	derivatized	33
H154A	4-NPPC12, (+)-pentazocine	no significant effect	33
E158G	haloperidol	no significant effect	11
E163G	haloperidol	no significant effect	11
M170C	[ <sup>3</sup> H]DTG	no significant effect	33
E172G	haloperidol	large	11
E172D	(+)-pentazocine	large	30
Y173A	(+)-pentazocine	large	30
Y173S <sup>b</sup>	cholesterol	large	72
R175A	(+)-pentazocine	large	30
T181A	(+)-pentazocine	no significant effect	30
F184A	(+)-pentazocine	no significant effect	30
D188G	haloperidol	no significant effect	11
D188	[ <sup>125</sup> I]IACoc	derivatized	27
T189A	(+)-pentazocine	no significant effect	30
F191A	(+)-pentazocine	no significant effect	30
D195G	haloperidol	no significant effect	11
Y201S/Y206S <sup>b</sup>	cholesterol	large	72
E213G	(+)-pentazocine	no significant effect	11
D222G	haloperidol	no significant effect	11
Deletion of 179-223	[ <sup>125</sup> I]IACoc	large	27
Deletion of 189-223	[ <sup>125</sup> I]IACoc	large	27
Deletion of 199-223	[ <sup>125</sup> I]IACoc	large	27
Deletion of 209-223	[ <sup>125</sup> I]IACoc	large	27
Deletion of 219-223	[ <sup>125</sup> I]IACoc	no significant effect	27
Deletion of 201-223	(+)-pentazocine	large	30
Deletion of 209-223	(+)-pentazocine	large	30
Deletion of 217-223	(+)-pentazocine	moderate	30
Deletion of 119-149	haloperidol	large	71

<sup>a</sup>Abbreviations: [<sup>125</sup>I]IABM, [methanesulfonothioic acid, S-((4-(4-amino-3-[<sup>125</sup>I]iodobenzoyl)phenyl)methyl) ester; 4-NPPC12, N [3-(4-nitrophenyl)propyl] N dodecylamine; [<sup>3</sup>H]DTG, 1,3-di(2-tolyl)guanidine; [<sup>125</sup>I]IACoc, methyl-3-(4-azido-3-[<sup>125</sup>I]iodo-benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate; (+)-PPP, (+)-1-propyl-3-(3-hydroxyphenyl)piperidine.

<sup>b</sup>In this study, the effects of substitutions on cholesterol binding were tested in the context of 20 amino acid synthetic peptides rather than the full-length receptor.

## Figure legends

**Figure 1:** Local sequence alignments of the *ERG2* family. Selected S1R sequences are aligned with the fungal  $\Delta 8 \rightarrow \Delta 7$  sterol isomerases and four related sequences from  $\gamma$ - or  $\delta$ -proteobacteria for the TM1 region (top; residues 1-40 of human S1R), the SBDL1 region (middle; residues 81-120 of human S1R), and the SBDL2 region (residues 176-205 of human S1R). The local alignments were extracted from an alignment of the full-length sequences carried out with T-Coffee<sup>73</sup>. For the TM1 region, the unconserved inserts of residues 7-41, 8-23 and 6-20 of *N. crassa*, *D. discoideum*, and *U. maydis*, respectively, were removed prior to aligning. Below each alignment is indicated whether the position is strictly conserved (\*), conserved as amino acids of strongly similar properties (:), or conserved as amino acids of weakly similar properties (.). Amino acid names are colored according to whether they are nonpolar (red), polar uncharged (green), polar positively charged (magenta), or polar negatively charged (blue).

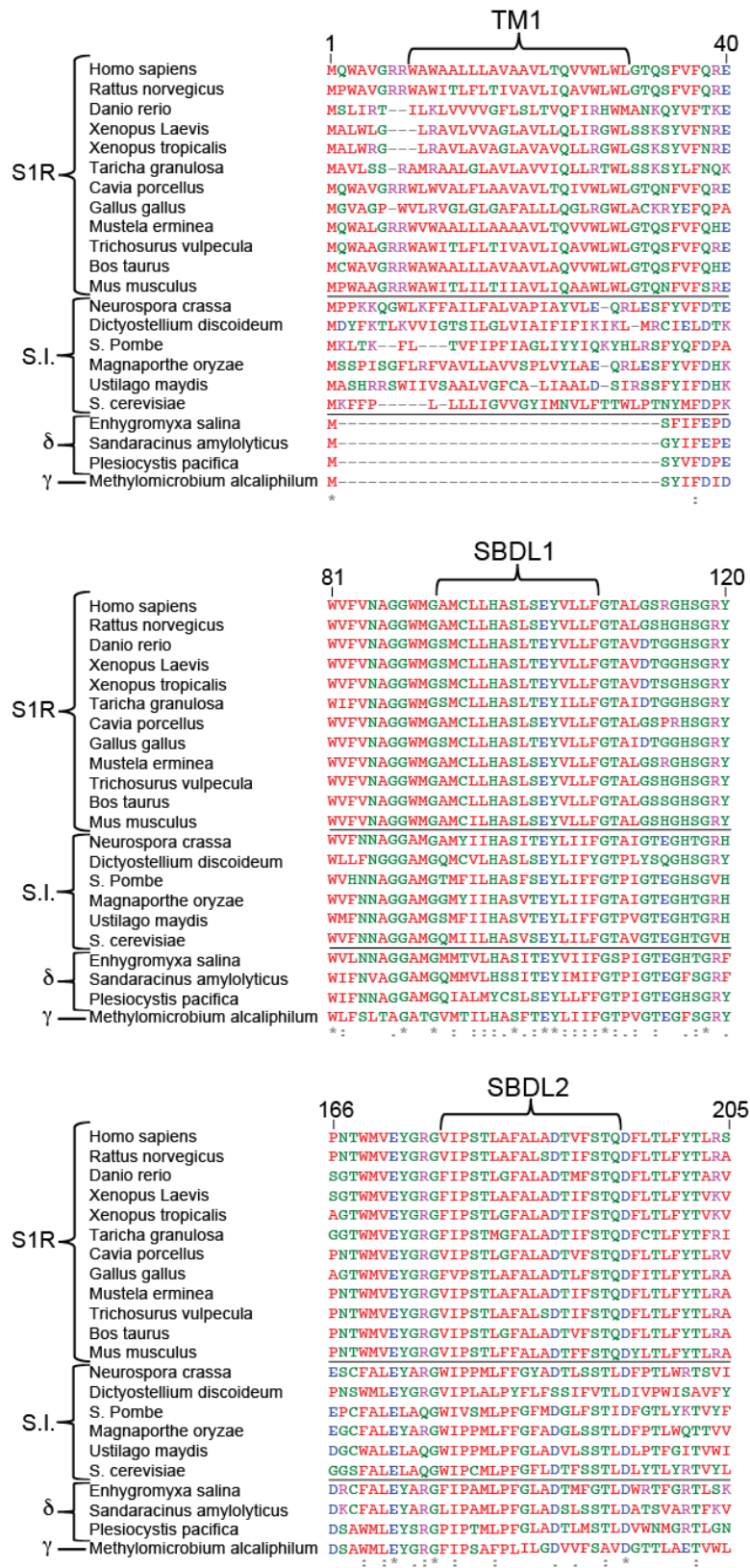
**Figure 2:** Structural overview and secondary structure topology of human S1R. **(A)** Cartoon diagram of the homotrimeric structure of S1R (PDB: 5HK1). Left: view down the symmetry axis from the perspective of the membrane. For clarity, the transmembrane helices have been removed. Right: side view from within the membrane (shown as two, gray, horizontal lines). The ligand PD144418 is represented as black sticks. **(B)** Cartoon diagram of a single subunit bound to the ligand PD144418 (white van der Waals spheres) (PDB: 5HK1). The transmembrane helix is colored gray, and the rest of the cartoon is colored from blue (N-terminal) to red (C-terminal). Strand numbering and helix lettering are identical to that of the topology diagram. **(C)** Topology diagram illustrating the S1R fold in which two sheets are flanked on each end by helical regions. The two sheets are connected by a hydrogen bond between the backbone amide of Met90 at the N-terminus of strand 2 and the sidechain hydroxyl of Ser113 at the N-terminus of strand 4. The topology diagram was adapted from a Pro-Origami output<sup>74</sup>. **(D)** Cartoon diagram of S1R from the perspective of the membrane in which the TM helix is oriented toward the viewer. Residues 183-184, 186-187 and 189 in helix D and residues 197-200 and 202-204 in helix E that have been shown to interact strongly with detergent<sup>25</sup> are shown as sticks and shaded green. All structural figures were generated using PyMol (Schrödinger, LLC).

**Figure 3:** **(A)** Cartoon diagram from the perspective of the membrane in which the TM helix is oriented toward the viewer. The SBDL regions 1 (residues 91-109) and 2 (residues 176-

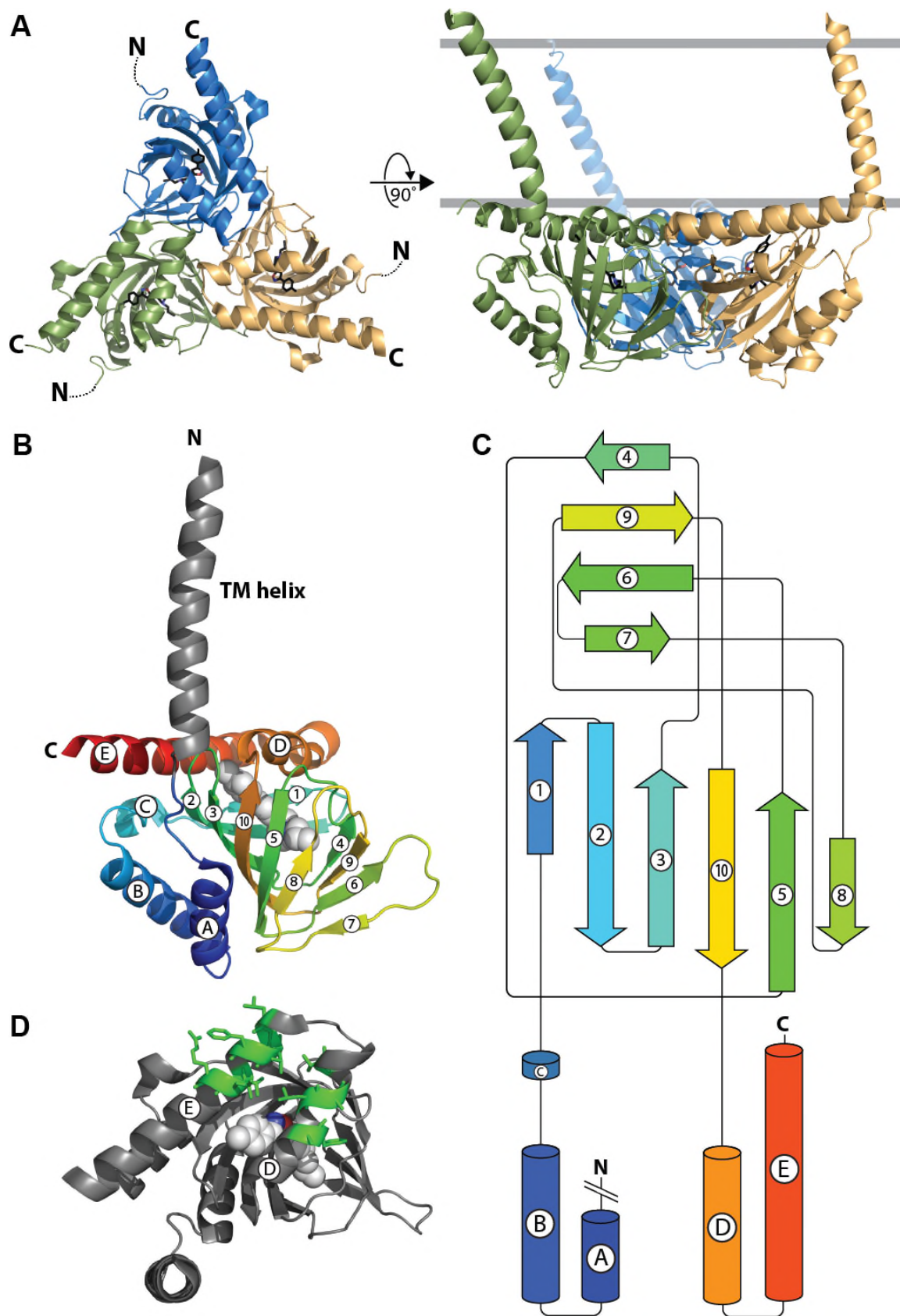
194) that were predicted from sequence analysis to be important in ligand binding<sup>26, 27</sup> are shaded in cyan. **(B)** Mapping of positions for which information on drug binding has been tested onto a subunit from PDB 5HK1 (**Table 1**). Positions at which substitutions have been shown to have large, moderate, or no significant effect on ligand binding are colored red, yellow, or blue, respectively. The positions of residues C94, H154, and D188, which have been shown to be derivatised by photoreactive probes are colored magenta. For clarity, strands 4, 6, and 9 of the small  $\beta$ -sheet have been removed. The ligand, PD144418, is represented as black sticks. The predicted position of the membrane is indicated by a gray line.

**Figure 4:** S1R ligand binding site and pharmacophore models. **(A)** Pharmacophore model derived from N-substituted phenylalkylamines<sup>36, 37</sup>. Schematic based on<sup>37</sup>. **(B)** A schematic diagram of the S1R residues lining the binding pocket for PD144418 based on PDB file 5HK1. The indicated distances are from C $\delta$  of E172 to N4 of PD144418, from an O $\delta$  of D126 to an O $\epsilon$  of E172, and from OH of Y103 to an O $\epsilon$  of E172. The leftmost residue, H154, is in  $\beta$ -strand 8 at the narrower end of the  $\beta$ -barred-like fold, whereas the rightmost residue, Y206, is in the membrane-proximal helix E. The figure is an adaptation of a LigPlot output<sup>75</sup>. **(C)** Pharmacophore model of Laggner *et al.* mapped onto fenpropimorph<sup>43</sup>. **(D)** Pharmacophore model of Zampieri *et al.* mapped onto a benzooxazolone derivative<sup>46</sup>.

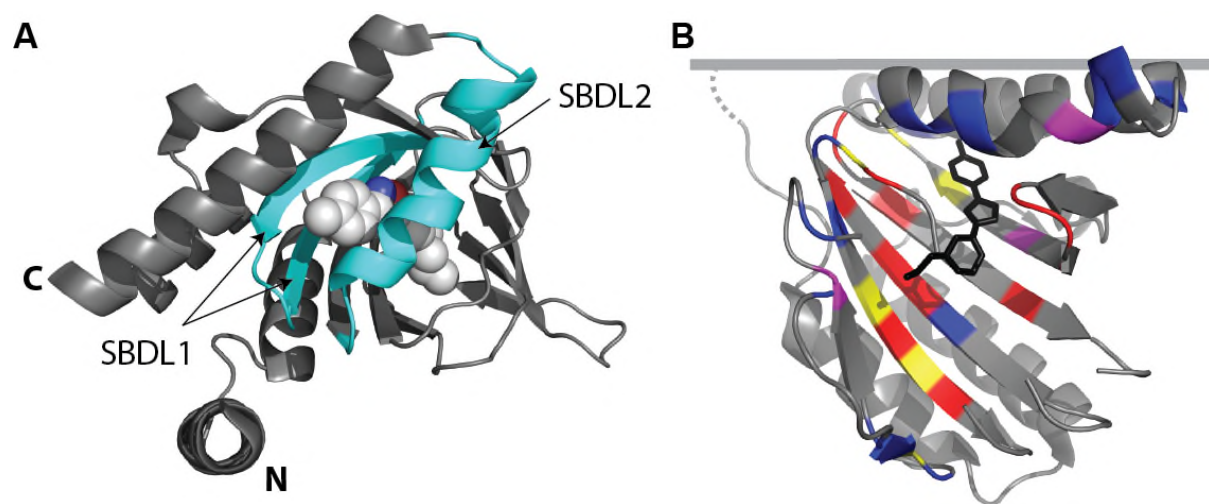
Figure 1



**Figure 2**



**Figure 3**





**Figure 4**

