Detection of New Biased Agonists for the Serotonin 5-HT<sub>2A</sub> Receptor: Modeling and Experimental Validation

Maria Martí-Solano, Alba Iglesias, Gianni de Fabritiis, Ferran Sanz, José Brea, M. Isabel Loza, Manuel Pastor, and Jana Selent

Research Programme on Biomedical Informatics, Department of Experimental and Health Sciences, Pompeu Fabra University, Hospital del Mar Medical Research Institute, Barcelona, Spain (M.M.-S., G.F., F.S., M.P., J.S.), and Department of Pharmacology, Institute of Industrial Pharmacy, Faculty of Pharmacy, Santiago de Compostela University, Santiago de Compostela, Spain (A.I., J.B., M.I.L.)

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ABSTRACT

Detection of biased agonists for the serotonin 5-HT<sub>2A</sub> receptor can guide the discovery of safer and more efficient antipsychotic drugs. However, the rational design of such drugs has been hampered by the difficulty detecting the impact of small structural changes on signaling bias. To overcome these difficulties, we characterized the dynamics of ligand-receptor interactions of known biased and balanced agonists using molecular dynamics simulations. Our analysis revealed that interactions with residues S5.46 and N6.55 discriminate compounds with different functional selectivity. Based on our computational predictions, we selected three derivatives of the natural balanced ligand serotonin and experimentally validated their ability to act as biased agonists. Remarkably, our approach yielded compounds promoting an unprecedented level of signaling bias at the 5-HT<sub>2A</sub> receptor, which could help interrogate the importance of particular pathways in conditions like schizophrenia.

Introduction

Serotonin 5-HT<sub>2A</sub> receptors are G protein–coupled receptors (GPCRs) targeted by hallucinogenic drugs of abuse (Nichols, 2004) as well as second-generation antipsychotic drugs (Meltzer, 1999), which function as antagonists at these receptors (González-Maeso and Sealfon, 2009). However, the basis of serotonin 5-HT<sub>2A</sub> receptor functioning is still not fully understood. Past studies on this receptor revealed that it can be differentially modulated by diverse agonists. Specifically, 5-HT<sub>2A</sub> receptors were one of the first GPCRs for which functional selectivity was described (Berg et al., 1998). Implication of these two pathways in processes, such as the generation of hallucinogenic effects, is still not completely understood (González-Maeso et al., 2007). In parallel, recent studies on the inactivation of the 5-HT<sub>2A</sub> receptor by antipsychotic drugs have pointed to an unwanted silencing effect on the transcription of the mGlu2 receptor, suggesting that full receptor inactivation could be counterproductive for the treatment of schizophrenia (Kurita et al., 2012). For these reasons, obtaining biased agonists capable of selectively activating each of these signaling pathways could help explore to which extent they are implicated in the aforementioned pathophysiological processes. This knowledge...
could, in turn, suggest new strategies for the design of more efficient drugs targeting the 5-HT_{2A} receptor.

At present, however, the rational design of biased agonists is hampered by the fact that the structural basis of functional selectivity is not fully understood. The problem arises partially from the challenge of attributing structural differences of agonist binding to distinct signaling states of the same receptor, which can be relatively subtle. Given the ability of GPCRs to explore different activation states, a single static picture of an activated receptor may not be enough to characterize contacts with agonists that promote different types of signaling bias. Therefore, a dynamic view of ligand-receptor interactions could add important information to understand the phenomenon of biased agonism. Recent advances in molecular dynamics (MD) simulations, which are currently used to study processes, such as GPCR activation inactivation (Dror et al., 2011) or stabilization of different receptor populations by agonists and inverse agonists (Nygard et al., 2013), provide a powerful tool for analyzing the structural basis of biased agonism at an adequate structural and temporal resolution.

Our study aims to learn from the dynamics of ligand-receptor interaction of known biased agonists, and apply this knowledge to the design of ligands with a tailored biased signaling profile. To assess this approach, we have applied extensive MD simulations with an accumulated time of 10 microseconds to study the structural determinants of biased agonism at the 5-HT_{2A} receptor from a dynamic perspective (see Supplemental Table 1 for details). Our study revealed ligand features as well as relevant hotspots within ligand interaction profiles that are related to signaling bias. By exploiting this structural knowledge, we have predicted compounds with the potential to behave as biased agonists. Importantly, experimental characterization of these compounds verified their biased nature and confirms the value of MD simulations for rationally detecting ligands promoting tailored signaling outcomes, which can provide a starting point for the design of new antipsychotic therapies.

**Materials and Methods**

**Homology Modeling and Ligand Docking.** Even if X-ray crystal structures of serotonin receptors have become recently available (PDB IDs 4IAR and 4IB4), the fact that these receptors have been crystallized in intermediate activation states (Wacker et al., 2013), which cannot accommodate a G protein (see Supplemental Fig. 6), led us to select the structure of the $\beta_2$-adrenergic receptor in complex with $G_s$ as the starting template to ensure simulation of a fully activated receptor. The modeling protocol included alignment of the sequence of the serotonin 5-HT_{2A} receptor to the one on the $\beta_2$-adrenergic receptor in complex with $G_s$ (PDB ID 3SN6) using Molecular Operating Environment (MOE) software (http://www.chemcomp.com/software.htm). A structural model of the receptor was then built using MODELER software (Laskowski et al., 1993). The resulting structures were optimized using the AMBER12: extended Hückel theory force field (Case, 2012) in the MOE software. The stereochemical quality of the model was evaluated with PROCHECK (Laskowski et al., 1993). After being analyzed with MoKa (Milletti and Vulpetti, 2010), the ligands were docked using GOLD software (Vordon et al., 2009) and the conformational space of the ligands was explored with the low mode search function of MOE using the AMBER12:extended Hückel theory force field (for further methodological information please refer to the Supplemental Materials and Methods).

**System Preparation and Molecular Dynamics Simulations.** Complexes resulting from the previous step were subsequently used to build the initial models for MD simulations [Protein Data Bank (PDB) ID 4IAR or 4IB4), the fact that these receptors have been crystallized in intermediate activation states (Wacker et al., 2013), which cannot accommodate a G protein (see Supplemental Fig. 6), led us to select the structure of the 5-HT_{2A} receptor in complex with Gs (PDB ID 4IAR) as the starting template to ensure simulation of a fully activated receptor. The modeling protocol included alignment of the sequence of the serotonin 5-HT_{2A} receptor to the one on the $\beta_2$-adrenergic receptor in complex with $G_s$ (PDB ID 3SN6) using Molecular Operating Environment (MOE) software (http://www.chemcomp.com/software.htm). A structural model of the receptor was then built using MODELER software (Laskowski et al., 1993). The resulting structures were optimized using the AMBER12: extended Hückel theory force field (Case, 2012) in the MOE software. The stereochemical quality of the model was evaluated with PROCHECK (Laskowski et al., 1993). After being analyzed with MoKa (Milletti and Vulpetti, 2010), the ligands were docked using GOLD software (Vordon et al., 2009) and the conformational space of the ligands was explored with the low mode search function of MOE using the AMBER12:extended Hückel theory force field (for further methodological information please refer to the Supplemental Materials and Methods).

**Drugs and Reagents.** [H]myo-inositol (20.3 Ci/mmol) and [3H]arachidonic acid (57.1 mCi/mmol) were purchased from PerkinElmer Life Science (Waltham, MA). 3-(2-aminoethyl)-1-methyl-1H-indol-5-ol was purchased from AstraZeneca (London, U.K.). 5-HT_{2A} receptors were isolated from CHO cells expressing the 5-HT_{2A} receptor (Sigma-Aldrich). 2-(5-nitro-1H-indol-3-yl)ethanamine was purchased from Sigma-Aldrich.

**Cell Culture.** Chinese hamster ovary cells stably expressing the human 5-HT_{2A} receptor at a density of $\approx 200$ fmol/mg protein [CHO-FA4 cells previously used in 2C-N biased agonism determination by Narayan et al. (2007)] were maintained in standard tissue culture plates (150 mm in diameter) in Dulbecco's modified Eagle's medium (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% glutamine (Gibco/Life Technologies, Grand Island, NY), 100 U/ml penicillin/100 μg/ml streptomycin (Sigma-Aldrich), and 300 μg/ml hygromycin (Invitrogen/Life Technologies, Grand Island, NY). Cells were grown at 37°C in a 5% CO₂ humidified atmosphere.

**Competition Binding in Human 5-HT_{2A} Receptors.** Serotonin 5-HT_{2A} receptor competition binding experiments were carried out in membranes from CHO-5HT_{2A} cells. On the day of the assay, membranes were defrosted and resuspended in binding buffer (50 mM Tris-HCl, pH 7.5). Each reaction well of a 96-well plate, prepared in duplicate, contained 80 μg of protein, 1 μM [3H]ketanserin (50.3 Ci/mmol; PerkinElmer Life Science), and compounds in various concentrations. Non-specific binding was determined in the presence of 1 μM methysergide (Sigma-Aldrich). The reaction mixture was incubated at 37°C for 30 minutes, after which samples were transferred to a multiscreen FB 96-well plate (Millipore, Madrid, Spain), filtered, and washed six times with 250 μl of wash buffer (50 mM Tris-HCl, pH 6.6) before measuring in a microplate beta scintillation counter (Microbeta TriLux, PerkinElmer, Madrid, Spain).

**Measurement of IP Accumulation and AA Release in CHO-FA4 Cells Expressing 5-HT_{2A} Receptors.** Cells were seeded into 96-well tissue culture plates at a density of $2 \times 10^4$ cells/cm². After
24 hours, the medium was replaced by a serum-free medium with 10 μCi/ml [14C]arachidonic acid (57.1 μCi/mmol) for 4 hours at 37°C. Measurement of IP accumulation and AA release were made simultaneously from the same well (Berg et al., 1998; 1999). After the labeling period, cells were washed for 10 minutes at 37°C with Hanks’ balanced salt solution supplemented with 20 mM HEPES, 20 mM LiCl, and 2% fatty acid free bovine serum albumin (experimental medium). After washing, cells were incubated for 20 minutes with an experimental medium at 37°C containing vehicle or the indicated concentrations of drugs. At the end of the incubation time, aliquots of 90 μl of media were added to flexiplate with 150 μl of OphthiPhase for the measurement of [14C], which corresponds to AA release. The remaining medium was discarded, and 200 μl of 100 mM formic acid was added to the cells for 30 minutes at 4°C. Aliquots of 20 μl were added to flexiplate with 80 μl of a solution RNA binding ytrium silicate scintillation proximity assay beads for measuring accumulation of [1H]-IPs from the cells (IP1, IP2, and IP3, which are collectively referred to as IP). Radioactivity was quantified with a liquid scintillation counter WALLAC MicrobetaTriLux 1450-023 (PerkinElmer). The same procedure was used in a CHO wild-type cell line to assess dependency on the 5-HT2A receptor for AA and IP (PerkinElmer). This comparison has proven to be useful, provided that the ligand is withheld at the orthosteric binding pocket (Fig. 1, middle panel). Both ligands establish previously known interactions with the receptor and adopt a general conformation that is in line with previous serotonin binding models (Ebersole et al., 2003). For instance, they form a well described salt bridge between their positively charged nitrogen and the carboxylate of residue D3.32 [residue numbers follow the Ballesteros-Weinstein numbering scheme (Ballesteros and Weinstein, 1995)]. In addition, they establish common hydrophobic contacts between their aromatic regions and residue V3.33. In general terms, taking into account the complete interaction list of both ligands, it would not be possible to establish a differential interaction pattern. However, if we analyze the preferred interactions of both ligands over the whole simulation time, we can find interesting differences between both compounds. Hence, considering the top five interactions for each ligand (Fig. 1, bottom panel; Supplemental Fig. 1), the balanced natural ligand, serotonin, adopts two main stabilizing interactions in the form of two hydrogen bonds. One hydrogen bond is formed between the nitrogen of its indole ring and residue S5.46, whereas the other one is established between its hydroxyl substituent and residue N6.55. It is worth mentioning that N6.55 can also form a hydrogen bond with residue S5.43. Previous experimental evidence suggests that S5.43 is able to establish indirect interactions with different serotonergic agonists (Braden and Nichols, 2007). This would be in line with our ligand-binding mode, in which S5.43 does not show direct contacts with serotonin but indirect ones via N6.55. In contrast to serotonin, the biased compound 2C-N enters deeply into the receptor and interacts frequently with residue F6.51. Besides, the methoxy substituent present in this compound reaches higher toward the extracellular receptor opening and interacts with residue V5.40 of helix 5. Interestingly, within its top five interactions, we find that 2C-N is capable of forming a contact between its nitro group and residue N6.55 in helix 6, which is also observed for serotonin, the natural ligand (Fig. 1, bottom left panel). This observation suggested that interaction with N6.55 could be responsible for the activation of the AA pathway, as both serotonin and 2C-N interact with this residue and promote AA signaling. In this sense, these results point to position 6.55 as a possible hotspot determining AA over IP signaling. This is in line with site-directed mutagenesis studies at position 6.55 in other amine GPCRs. These studies revealed the influence of this position on biased signaling related to differential G protein coupling (Tschammer et al., 2011; Fowler et al., 2012). In parallel, the finding that serotonin establishes an interaction with residue S5.46 in helix 5, which is not seen in the dynamic binding profile of 2C-N, could justify the biased nature of the latter. Mutations in this position in receptors transfected in HEK293 cells, which have shown somewhat conflicting results regarding the binding mode of different

### Results

Assessing the Interaction Preferences of Known Compounds. To characterize the structural determinants of biased signaling at the 5-HT2A receptor, we started analyzing two representative compounds: serotonin, the natural ligand, which produces a balanced response for the two studied pathways, and 2C-N, a compound capable of partially stimulating AA release but lacking efficacy for IP accumulation (Moya et al., 2007). Both compounds, the natural ligand serotonin (used as a control for balanced agonism) and 2C-N, were docked into a fully activated model of the serotonin 5-HT2A receptor. Notably, the modeling approach used to obtain these ligand-receptor complexes, which is detailed in the Materials and Methods section, has previously proven to be highly effective in predicting high-resolution ligand-receptor complexes for related targets (Obiol-Pardo et al., 2011). The resulting complexes were embedded into a hydrated lipid bilayer, ionized to a physiologic concentration and subjected to extensive molecular dynamic simulations. Given the importance of appropriately sampling the ligand-receptor conformational space and retaining an activated receptor state for the study of biased agonism, we prioritized the use of independent replicates over the study of single prolonged simulations, which would have likely resulted in receptor inactivation, as observed for other GPCRs (Dror et al., 2011). In addition, simulations in which the receptor progressed to a fully inactivated state (assessed by ionic lock closure) were discarded from the analysis. Ultimately, the resulting simulations used for both ligands consist of eight independent replicates per ligand-receptor system, amounting to a total simulation time of 4 microseconds (see Supplemental Table 1). A structural analysis of the simulation reveals that the studied compounds can sample several positions within the orthosteric binding pocket (Fig. 1, middle panel). Both ligands establish previously known interactions with the receptor and adopt a general conformation that is in line with previous serotonin binding models (Ebersole et al., 2003). For instance, they form a well described salt bridge between their positively charged nitrogen and the carboxylate of residue D3.32 [residue numbers follow the Ballesteros-Weinstein numbering scheme (Ballesteros and Weinstein, 1995)]. In addition, they establish common hydrophobic contacts between their aromatic regions and residue V3.33. In general terms, taking into account the complete interaction list of both ligands, it would not be possible to establish a differential interaction pattern. However, if we analyze the preferred interactions of both ligands over the whole simulation time, we can find interesting differences between both compounds. Hence, considering the top five interactions for each ligand (Fig. 1, bottom panel; Supplemental Fig. 1), the balanced natural ligand, serotonin, adopts two main stabilizing interactions in the form of two hydrogen bonds. One hydrogen bond is formed between the nitrogen of its indole ring and residue S5.46, whereas the other one is established between its hydroxyl substituent and residue N6.55. It is worth mentioning that N6.55 can also form a hydrogen bond with residue S5.43. Previous experimental evidence suggests that S5.43 is able to establish indirect interactions with different serotonergic agonists (Braden and Nichols, 2007). This would be in line with our ligand-binding mode, in which S5.43 does not show direct contacts with serotonin but indirect ones via N6.55. 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These studies revealed the influence of this position on biased signaling related to differential G protein coupling (Tschammer et al., 2011; Fowler et al., 2012). In parallel, the finding that serotonin establishes an interaction with residue S5.46 in helix 5, which is not seen in the dynamic binding profile of 2C-N, could justify the biased nature of the latter. Mutations in this position in receptors transfected in HEK293 cells, which have shown somewhat conflicting results regarding the binding mode of different
tryptamines, highlight the importance of this interaction in the case of serotonin and call for a deeper characterization in our studied system (Braden and Nichols, 2007).

**Considering N6.55 Versus S5.46 Interaction Preferences to Propose New Biased Agonists.** Taken together, observations on the binding preferences of known balanced and biased agonists, and especially of the importance of interaction with residues N6.55 and S5.46, led us to suggest that biased agonism at the 5-HT$_{2A}$ receptor is determined as follows: ligand interaction with residue N6.55 in helix 6 favors the stabilization of receptor conformations with a preference to signal through the AA pathway, while interaction with S5.46 in helix 5 is responsible for facilitating signaling through the IP pathway. At this point, the most challenging task was to apply this structural knowledge for the experimental detection of new biased agonists. Such detection would represent an important milestone to validate our previous observations based on molecular dynamics simulations. We hypothesized that based on the above defined requirements, we could introduce structural modifications into the balanced natural agonist serotonin, turning it into a biased compound with tailored signaling behavior. To test this hypothesis, we searched for novel, commercially available, and previously uncharacterized ligands for biased agonism that contain a tryptamine scaffold, with the potential to interact with residues N6.55 or S5.46. Our search yielded three interesting compounds. The first selected compound is MetI (Fig. 2, upper panel). Compared with serotonin, this compound has a methyl substitution at the amine of the indol group, which, in principle, would diminish the capacity for hydrogen bonding with residue S5.46 in helix 5, but would still allow interaction with N6.55, therefore promoting AA over IP signaling. The second candidate, MetT (Fig. 2, upper panel), has a methyl substitution in the position occupied by the hydroxyl group in

![Serotonin and 2C-N](image)
serotonin. According to our hypothesis, this compound should show a decreased ability to form a hydrogen bond with residue N6.55, hence making it a biased agonist by promoting IP over AA signaling. Finally, to assess in a more refined way the ligand determinants related to functional selectivity, we selected a third compound, namely, NitroI (Fig. 2, upper right panel). This last compound preserves the amine of the indol group found in serotonin but has a nitro group substitution in the position occupied by the hydroxyl group of the natural ligand. In this way, this compound allows assessment of the importance of the nitro group present in 2C-N for interaction with residue N6.55. If the effect of the nitro group interaction is equivalent to the one established by the hydroxyl group of serotonin, NitroI should be able to signal through both pathways.

Upon selection of these three new biased agonist candidates, we undertook a new set of MD simulations to characterize their behavior inside of the serotonin 5-HT\textsubscript{2A} receptor binding pocket. We conducted the same protocol as the one previously applied for serotonin and 2C-N. The conformational space sampled by all three compounds is shown in the middle panel of Fig. 2. As in the case of serotonin and 2C-N, considering an extended list of ligand-receptor contacts (Supplemental Fig. 2) does not allow us to discriminate differential interaction patterns among the proposed biased ligands. Notably, a structural analysis of the overall receptor conformational space of the 5-HT\textsubscript{2A} receptor in complex with our studied ligands (Supplemental Fig. 3), despite showing some differences, does not allow discrimination of different signaling signatures either. Conversely, analysis of the top five ligand-receptor interactions reveals some expected differences in ligand-interaction preferences. In detail, main interactions, such as the salt bridge between the protonated nitrogen and D3.32 as well as hydrophobic contacts with V3.33, were observed among the selected compounds (Fig. 2, bottom panel; Supplemental Fig. 2). Assessment of the top five interactions for compounds MetI and MetT also shows differences in interaction with defined hotspots for biased signaling. In this context, our simulations reveal a preference for MetI to interact with residue N6.55 (Fig. 2, left bottom panel). This behavior is in agreement with our initial prediction that MetI should especially promote signaling through the AA pathway.

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Fig. 2. Analysis of the dynamic binding profile of potential biased ligands. Structures of the proposed biased agonists MetI (purple), MetT (yellow), and NitroI (magenta) (upper panels). Conformational space explored by each ligand as a superposition of 1 every 20 frames per trajectory (middle panels). Analysis of preferred ligand-receptor interactions (bottom panels). Key residues implicated in ligand-receptor hydrogen bonding are highlighted in red and bold. Hydrogen bonding is indicated as red dashed lines.
Conversely, MetT favors interaction with S5.46 in helix 5 (Fig. 2, middle panel), and therefore is predicted to stimulate signaling through the IP pathway. Interestingly, an unexpected behavior was observed for the third compound, NitroI. Even if this compound is able to interact at times with position N6.55 through its nitro group, analysis of the total simulation time shows that this is not within the top five interactions (Fig. 2, bottom panel) and that NitroI clearly favors hydrogen bonding with residue S5.46. According to our defined criteria for biased signaling, this observed interaction pattern indicates that NitroI should promote IP over AA signaling. All in all, our dynamic analysis predicts that MetI favors AA signaling, whereas MetT and NitroI signal preferentially via the IP pathway.

Experimental Validation of Biased Agonism for New Compounds. To validate the accuracy of our computational predictions, we experimentally determined their levels of signaling for the AA and IP pathway (Fig. 3; Table 1). In the first step, we confirmed that MetI, MetT, and NitroI bind specifically to the 5-HT2A receptor, with binding affinity constants ($K_i$) of 3.25, 0.86, and 2.05 μM, respectively (Supplemental Fig. 4). Besides that, AA and IP stimulation is not observed in the parental cell line either in the presence of serotonin or the new tested compounds (Supplemental Fig. 5), indicating that stimulation of these pathways depends on ligand binding to the 5-HT2A receptor. Regarding functional selectivity, in line with our computational prediction, our first tested compound, MetI, shows a preference to signal through the AA pathway over the IP one (Fig. 3; Table 1). This can be deduced from calculating its bias factor, which quantifies the relative stabilization of one signaling state over another compared with the reference agonist (Rajagopal et al., 2011) (please refer to the Materials and Methods section for a description of ligand bias calculation). Comparison of MetI with the natural ligand serotonin gives a bias factor of 1.77, indicating that MetI activates AA 17.7 times better over the IP pathway than serotonin. This first validation clearly demonstrates the ability of our model to rationally tune the balanced signaling stimulated by serotonin into an AA signaling preference. Even more striking are the results obtained for MetT. On top of showing the predicted bias for IP signaling, this compound does not have any detectable ability to promote signaling through the AA pathway at our tested concentrations, thus behaving as a highly biased agonist for the IP pathway (Table 1). NitroI also follows its predicted pattern, and, remarkably, it is capable of behaving as a full agonist for IP signaling at a nanomolar scale while not triggering the AA pathway. To our knowledge, this level of bias is unprecedented at this receptor and would make this last compound a particularly interesting tool to explore serotonin 5-HT2A receptor pharmacology.

Discussion

In our work, we have used extensive MD simulations to learn from the dynamics of ligand-receptor interactions of biased agonists. This dynamic insight provides a thorough sampling of ligand-binding preferences capable of discriminating different types of receptor agonists. In our experience, this discrimination would have been difficult if only their docking poses had been considered. Our simulations highlight the importance of contacts with particular receptor hotspots for biased agonism, namely, N6.55 in the case of AA signaling.
and S5.46 in the case of IP signaling. Based on this knowledge, we have discovered new biased ligands of unprecendented efficacy by tuning the structure of the balanced natural ligand serotonin. Experimental validation of the proposed ligands has proven the power of characterizing dynamics of ligand-receptor interactions to obtain ligands with a tailored biased signaling profile. This study, however, poses interesting questions on the process of functional selectivity at the 5-HT₂A receptor that go beyond ligand-receptor interactions. Given that in this study we were only able to obtain compounds with very high levels of bias for IP signaling (MetT and NitrO), the question of how feasible it is to obtain this kind of agonist for the AA pathway remains. This could be a complicated mission, in case receptor conformations related to differential coupling overlap in such a way that when activating the AA pathway, there will always be a receptor population capable of triggering IP signaling. This calls for a deeper structural characterization of diverse receptor states coupled to specific signaling transducers. In parallel, the applicability of our model could be further extended by the incorporation of additional 5-HT₂A receptor agonists possessing significantly different chemical scaffolds than the ones considered in this work. Further experimental and computational studies will be needed to solve these questions. In particular, experimental structural information on receptors coupled to different G proteins would shed light on the overall receptor architecture required for differential coupling. This information would enrich studies like the current one, as interaction with different biased agonists in the absence of a G protein is not considered enough to stabilize particular receptor signaling states (Rasmussen et al., 2011; Thanawala et al., 2014). Nonetheless, results presented in this work highlight the potential of ligand-receptor dynamics simulations to rationalize biased signaling determinants. In our particular case, the obtained biased agonists could represent valuable tools to interrogate particular signaling pathways as well as inspire the development of new drug candidates, with improved efficacy and safety profiles for the treatment of conditions, such as schizophrenia.

**Authorship Contributions**

**Participated in research design:** Martí-Solano, Sanz, Brea, Pastor, Selent.

**Conducted experiments:** Martí-Solano, Iglesias, Selent.

**Contributed new reagents or analytic tools:** de Fabritiis, Selent.

**Performed data analysis:** Martí-Solano, Brea, Selent.

**Wrote or contributed to the writing of the manuscript:** Martí-Solano, Sanz, Brea, Loza, Pastor, Selent.

**References**


Case DA (2012) AMBER 12, University of California, San Francisco, CA.


**Address correspondence to:** Jana Selent, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, IMIM (Hospital del Mar Medical Research Institute), Dr. Aiguader 88, E-08003 Barcelona, Spain. E-mail: jana.selent@upf.edu or Manuel Pastor, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, IMIM (Hospital del Mar Medical Research Institute), Dr. Aiguader 88, E-08003 Barcelona, Spain. E-mail: manual.pastor@upf.edu
Supplemental information

Detection of new biased agonists for the serotonin 5-HT$_2A$ receptor: modeling and experimental validation

*Maria Martí-Solano, Alba Iglesias, Gianni de Fabritiis, Ferran Sanz, José Brea, M. Isabel Loza, Manuel Pastor, and Jana Selent*

Research Programme on Biomedical Informatics (GRIB), Department of Experimental and Health Sciences, Pompeu Fabra University, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain (M M-S, G de F, F S, M P, J S).

Department of Pharmacology, Institute of Industrial Pharmacy, Faculty of Pharmacy, Santiago de Compostela University, Santiago de Compostela, Spain (A I, J B, MI L).
Supplemental Materials and Methods

_Homology modeling_

In a first step the sequence of the serotonin 5-HT$_{2A}$ receptor was first retrieved from the UniProt database. In order to ensure a fully activated conformation, the crystal structure of the β2-adrenergic receptor in complex with Gs was used as a template (PDB code 3SN6). Both sequences were aligned using the MOE software (Molecular Operating Environment (MOE) software, http://www.chemcomp.com/software.htm). The resulting alignment was manually refined to ensure alignment of the highly conserved residues of the GPCR superfamily. Starting from the resulting alignment, the structural models of the receptors were built using the MODELLER software (Sali and Blundell, 1993). In this procedure, the conserved disulfide bond between residue C3.25 at the beginning of TM3 and cysteine 277 in ECL2 was taken into account and maintained as a constraint for geometric optimization. The best structures were selected from these candidates according to the MODELLER objective function and visual inspection. The resulting receptor structures were optimized by the AMBER12:EHT force field (Gerber and Müller, 1995; Case, 2012) using the molecular modeling program MOE (Molecular Operating Environment (MOE) software, http://www.chemcomp.com/software.htm). Then, the PROCHECK software (Laskowski et al., 1993) was used to assess the stereo chemical quality of the minimized structures, resulting in good quality parameters and an excellent distribution of phi and psi angles in the Ramachandran plot.
Ligand Docking and pose refinement

Ligands were docked into the 5-HT$_{2A}$ receptor using the GOLD software (Verdonk et al., 2003) defining a centroid point in residue D3.32 and expanding it 20 Å around this residue. One hundred genetic algorithm runs were submitted and further scored employing the ASP scoring function. The ligands were restricted to form a salt bridge between their positively charged nitrogen and the carboxylate of D3.32. The best poses from this docking procedure were used as input to explore the conformational space of the ligands with the Low Mode Search function of MOE, which is a short MD simulation using velocities with little kinetic energy on the high-frequency vibrational modes, using the AMBER12:EHT force field, Born solvation, 300 K and default settings. In a first step, the ligand, the side chains of the binding pocket (defined as residues 4.5 Å apart from the ligand) and the extracellular loop 2 were kept free while the rest of the receptor was fixed. In addition, the formation of a salt bridge between the positively charged nitrogen and the carboxylate of D3.32 was forced by fixing the distance between these two atoms. In a second step, this procedure was repeated but the whole system was kept fixed except for the ligand. The best solutions derived from this procedure were subjected to energy minimization by applying gradient minimization until the RMS gradient was lower than 0.001 kcal mol$^{-1}$Å$^{-1}$.

Molecular dynamics protocol

In a first step, each system was submitted to a minimization procedure for 3000 steps. In a second step, the system was equilibrated using the NPT ensemble with a target pressure equal to 1.01325 bar, a time-step of 2 fs and using the RATTLE algorithm for the hydrogen atoms. In this stage, the harmonic constraints applied to the heavy atoms of
the protein and ligand were progressively reduced until an elastic constant force equal to 0 kcal/mol and the temperature was increased to 300K. The purpose of this relaxation phase is to allow for a complete adjustment of membrane lipids to the receptor, thus filling non-physiological gaps between receptor and membrane lipids. All the simulations were conducted using the same non-bonded interaction parameters, with a cutoff of 9 Å, a smooth switching function of 7.5 Å and the non-bonded pair list set to 9 Å. The periodic boundary conditions were set to a size of 94x94x100, and for the long range electrostatics we used the PME methodology with a grid spacing of 1 Å. In a third step, production phases were performed using the NVT ensemble with aforementioned parameters but a time-step of 4 fs, and a hydrogen scaling factor of 4. This timestep is possible due to the implementation of the hydrogen mass repartitioning scheme in the ACEMD code (Feenstra, 1999).

Conformational space analysis of the whole receptor

The receptor trajectories of the 5 different simulated systems (derived from the simulations with serotonin, 2C-N, MetT, MetI and NitroI) were concatenated and aligned. Next, they were subjected to an all atom principal component analysis using the P traj package implemented in Amber using default conditions (Case, 2012).
**Supplemental Table S1.** Simulation details of each independently-run ligand-receptor system.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Replicates</th>
<th>Simulation time</th>
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<tbody>
<tr>
<td>Serotonin</td>
<td>8</td>
<td>250 ns</td>
</tr>
<tr>
<td>2C-N</td>
<td>8</td>
<td>250 ns</td>
</tr>
<tr>
<td>MetI</td>
<td>8</td>
<td>250 ns</td>
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<tr>
<td>MetT</td>
<td>8</td>
<td>250 ns</td>
</tr>
<tr>
<td>NitroI</td>
<td>8</td>
<td>250 ns</td>
</tr>
<tr>
<td>Total simulation time</td>
<td>10 µs</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Figure 1. Percentage of ligand-receptor interactions for known ligands. Residues represented in dark color correspond to top five interacting residues considering the whole accumulated simulation time (2 µs per system).

Supplemental Figure 2. Percentage of ligand-receptor interactions of potential biased ligands. Residues represented in dark color correspond to top five interacting residues considering the whole accumulated simulation time (2 µs per system).
Supplemental Figure 3. Principal component analysis (PCA) of the trajectories of the 5-HT2A receptor in complex with different ligands. A) Distribution in the first two principal components of unique snapshots of every receptor conformation in complex with: serotonin (orange) and 2C-N (beige). B) Distribution in the first two principal components of unique snapshots of every receptor conformation in complex with: serotonin (orange), 2C-N (beige) and MetI (purple). Newly selected compounds are plotted in 3 separate plots for clarity. C) Distribution in the first two principal components of unique snapshots of every receptor conformation in complex with: serotonin (orange), 2C-N (beige) and MetT (yellow). Newly selected compounds are plotted in 3 separate plots for clarity. D) Distribution in the first two principal components of unique snapshots of every receptor conformation in complex with: serotonin (orange), 2C-N (beige) and NitroI (magenta). Newly selected compounds are plotted in 3 separate plots for clarity. Analysis of the different plots points to a different exploration of conformational space by the receptor depending on the ligand bound to it. However, there is not a clear discrimination between compounds showing different bias.
Although compounds which potentiate AA over IP signaling (2C-N (beige) and MetI (purple)) compared to the ones that preferentially promote IP signaling (MetT (yellow) and NitroI (magenta)) show some differences in conformational space, their high degree of overlap does not allow a clear discrimination related to differential receptor signaling. This would be in line with observations that interaction with different biased agonists in the absence of a G protein may not be enough to stabilize particular receptor signaling states (Rasmussen et al., 2011; Thanawala et al., 2014).

Supplemental Figure 5. Concentration-response curves of 5-HT at CHO WT cell line measuring IP formation (left) and AA release (right). Points represent the mean±SD of three independent experiments.
Supplemental Figure 6. Structural comparison between available serotonergic crystal structures (1B and 2B receptors) and the β2-adrenergic receptor in complex with Gs. Superposition of the crystal structures of the three receptors (4IAR, 4IB4 and 3SN6) shows that the 1B and 2B receptors (cyan and lime) are in a partially active conformation. Unlike the β2-adrenergic receptor (gray), in which there is an outward tilt of helix 6 (red arrow), the serotonin receptor structures cannot accommodate the Ga subunit.
References

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