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Detection of New Biased Agonists for the Serotonin 5-HT_{2A} Receptor: Modeling and Experimental Validation[S]

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ABSTRACT

Detection of biased agonists for the serotonin 5-HT_{2A} 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_{2A} receptor, which could help interrogate the importance of particular pathways in conditions like schizophrenia.

Introduction

Serotonin 5-HT_{2A} 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_{2A} 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_{2A} receptors were one of the first GPCRs for which functional selectivity was described (Berg et al., 1998). Functional selectivity allows some GPCRs to preferentially signal through a signaling pathway over another when they interact with certain ligands, namely, biased agonists (Urban et al., 2007). Biased agonists are suggested to promote the stabilization of distinct receptor activation states, with a preference to couple to a given signal transducer and thus favor signaling through a particular pathway (Park, 2012). Ever since the first description of this class of compounds, they were considered likely drug candidates (Whalen et al., 2011). Biased agonists hold a big potential as new generation drugs with increased efficacy and safety (Martí-Solano et al., 2013) by modulating pathways implicated in disease, while sparing other nonrelated cellular processes regulated by the activation of the same receptor.

More than a decade ago, Berg et al. described the different ability of some ligands to trigger two independent signaling responses at the 5-HT_{2A} receptor: the accumulation of inositol phosphate (IP) and the release of arachidonic acid (AA) (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_{2A} 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

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ABBREVIATIONS: AA, arachidonic acid; GPCR, G protein–coupled receptor; IP, inositol phosphate; MD, molecular dynamics; MetI, 3-(aminoethyl) 1-methylindol-5-ol; MetT, 5-methyltryptamine; MOE, Molecular Operating Environment; NitroI, 5-nitro-1*H*-indole-3-ethanamine; 2C-N, 2,5-dimethoxy-4-nitrophenethylamine.

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 (Nygaard 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 β2-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 β2-adrenergic receptor in complex with G_s (PBD 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 (Latek et al., 2013). The resulting structures were optimized using the AMBER12: extended Hueckel 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 (Verdonk et al., 2003) and the conformational space of the ligands was explored with the low mode search function of MOE using the AMBER12:extended Hueckel 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 files of ligand–5-HT$_{2A}$ receptor complexes are available for serotonin (Data Supplement), 2,5-dimethoxy-4-nitrophenethylamine (2C-N) (Data Supplement), 5-methyltryptamine (MetT) (Data Supplement), 3-(aminoethyl)1-methylindol-5-ol (MetI) (Data Supplement), and 5-nitro-1*H*-indole-3-ethanamine (NitroI) (Data Supplement)]. First, the protonation state of titratable groups was predicted for a pH value of 7.4 based on PROPKA (Li et al., 2005) using the implemented prediction tool of the MOE package. Subsequently, to place both receptors into a membrane bilayer, a hole was generated by removing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine molecules of a pre-equilibrated palmitoyloleoylphosphatidylcholine bilayer generated using the CHARMM-GUI Membrane Builder (Jo et al., 2009). Lipids that were in close contact with the protein atoms (<1 Å distance from any protein atoms) were deleted. Finally, the coordinates for water and ions were generated using the solvate and autoionize modules of VMD 1.9.1 (Humphrey et al., 1996). The ionic strength was kept at 0.15 M by NaCl, and we used the TIP3 water model. The all-atom models of each system were generated by using the Amber03 force-field parameters, and the different ligands were parameterized using Antechamber from AmberTools 11 (Case, 2012). Simulations were performed using ACEMD (Harvey and De Fabritiis, 2009) following the protocol described in the Supplemental Materials and Methods. Simulations were performed for individually generated starting structures, and each ligand-receptor complex was run eight times for 250 nanoseconds. Analysis of ligand-receptor interactions was performed by considering residues at a distance smaller or equal to 3 Å of each ligand across the simulation time.

Drugs and Reagents. [^{3}H]myo-Inositol (20.3 Ci/mmol) and [^{14}C]arachidonic acid (57.1 mCi/mmol) were purchased from PerkinElmer Life Science (Waltham, MA). 3-(2-aminoethyl)-1-methyl-1*H*-indol-5-ol was purchased from Otava Ltd (Vaughan, ON, Canada). Serotonin, 5-methyltryptamine, and 2-(5-nitro-1*H*-indol -3-yl) ethanamine were purchased from Sigma-Aldrich (St. Louis, MO). RNA binding yttrium silicate scintillation proximity assay beads and the OptiPhaseSupermix cocktail were purchased from PerkinElmer Life Science. Albumin, Fraction V fatty acid free was purchased from Roche (Basel, Switzerland). All other reagents were purchased from Sigma-Aldrich.

Cell Culture. Chinese hamster ovary cells stably expressing the human 5-HT$_{2A}$ receptor at a density of ≈200 fmol/mg protein [CHO-FA4 cells previously used in 2C-N biased agonism determination by Moya et al. (2007)] were maintained in standard tissue culture plates (150 mm in diameter) in Dulbecco's modified Eagle's medium-F12 (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), 100 U/ml penicillin/0.1 mg/ml streptomycin (Sigma-Aldrich), and 300 μg/ml hygromycin (Invitrogen/Life Technologies, Grand Island, NY). Cells were grown at 37°C in a 5% CO_2 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 nM [^{3}H]ketanserin (50.3 Ci/mmol; PerkinElmer Life Science), and compounds in various concentrations. Nonspecific 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 2×10^4 cells/cm^2. After

24 hours, the medium was replaced by a serum-free medium with 10 μCi/ml [^{3}H]myo-inositol (20.3 Ci/mmol) for 24 hours and 0.2 μCi/ml [^{14}C]arachidonic acid (57.1 mCi/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 OpthiPhase for the measurement of [^{14}C], 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 yttrium silicate scintillation proximity assay beads for measuring accumulation of ^{3}H-IPs from the cells (IP_1, IP_2, and IP_3, 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-HT_{2A} receptor for AA and IP signaling activation.

Pharmacological Data Analysis. Stimulation response parameters were calculated with Prism 4.0 software (GraphPad Software, La Jolla, CA) applying a four parameter logistic equation. In the case of MetI, the fact that this ligand was not completely biased for one pathway led us to calculate a bias factor using an equiactive comparison. This method gives a good estimate of bias when the dissociation constant for a ligand is not known (Rajagopal et al., 2011). This comparison has proven to be useful, provided that the ligand is not a partial agonist or a strongly biased compound, as in the present case. Therefore, using this approach, comparison between the ligand and a reference (in this case, serotonin) provides a bias factor (β) for pathway P1 versus P2, which can be calculated as follows:

$$\beta = \log\left[\left(\frac{E_{max,P1}}{EC_{50,P1}}\frac{EC_{50,P2}}{E_{max,P2}}\right)_{lig} \times \left(\frac{E_{max,P2}}{EC_{50,P2}}\frac{EC_{50,P1}}{E_{max,P1}}\right)_{ref}\right]$$

Results

Assessing the Interaction Preferences of Known Compounds. To characterize the structural determinants of biased signaling at the 5-HT_{2A} 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-HT_{2A} 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. 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 aminergic 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

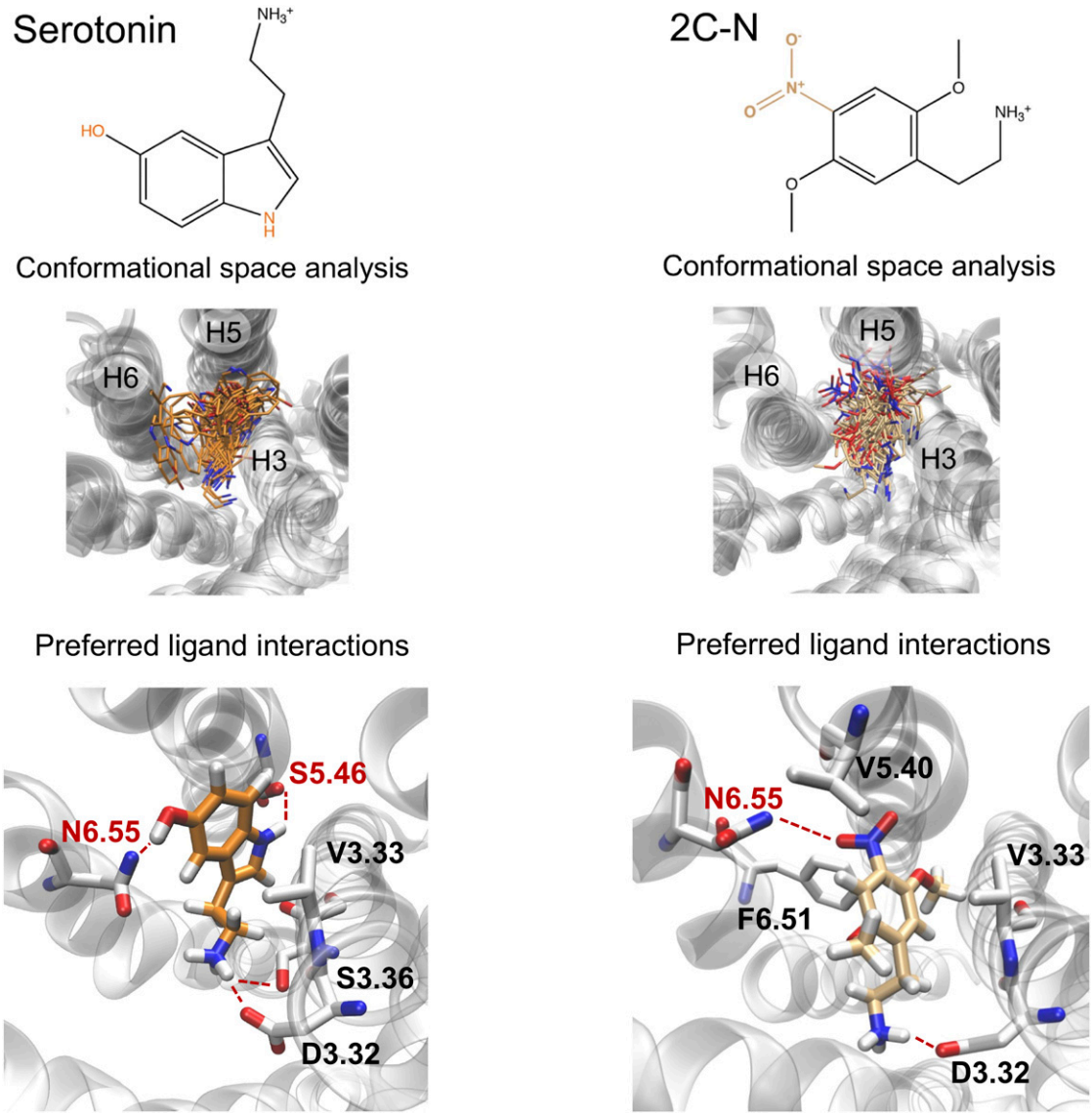

Fig. 1. Analysis of the dynamic binding profile of known biased and balanced ligands. Structures of the starting compounds serotonin (orange) and 2C-N (beige) (upper panels). Conformational space explored by each ligand as a superposition of 1 every 20 frames per trajectory (middle panels). Analysis 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.

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

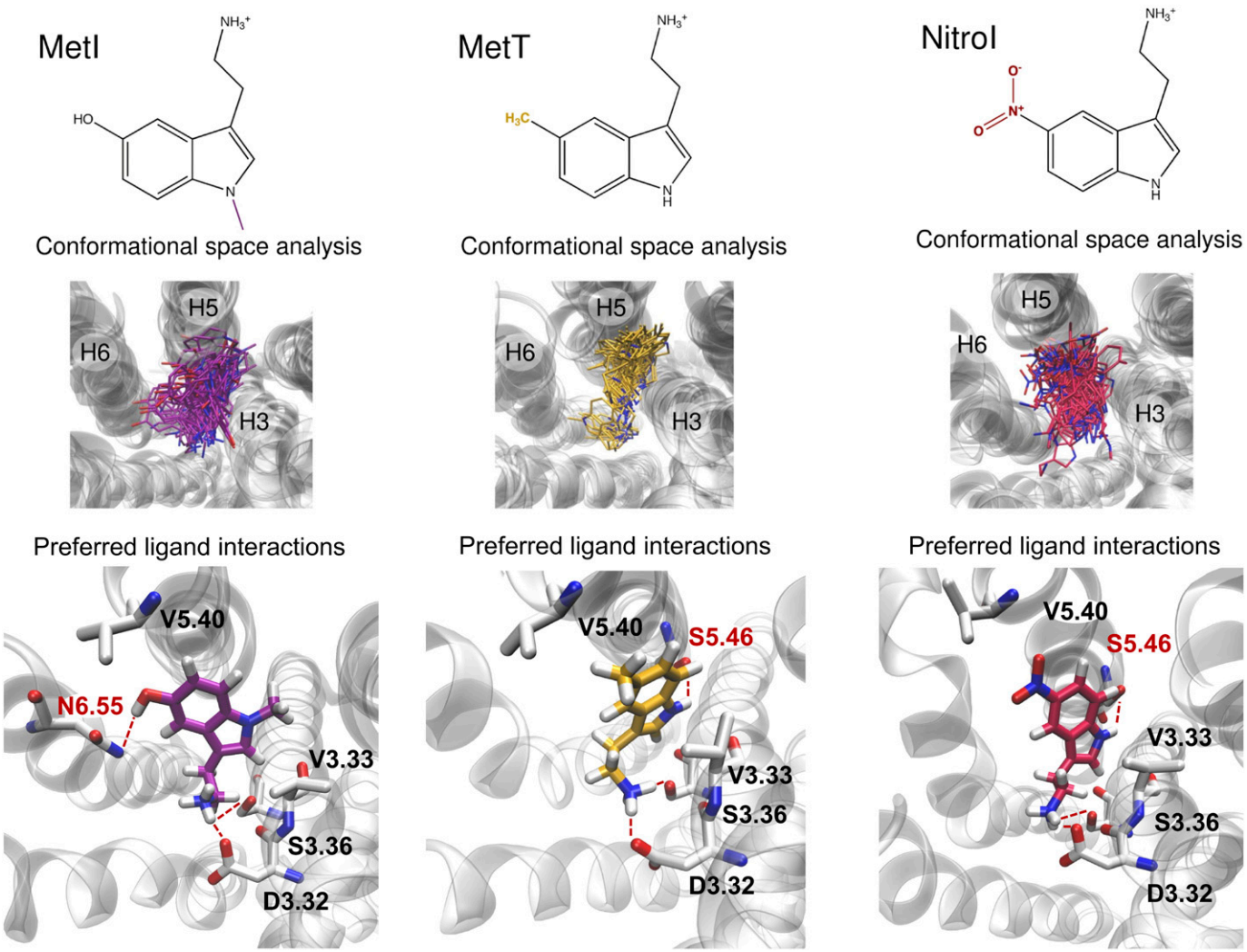

Fig. 2. Analysis of the dynamic binding profile of potential biased ligands. Structures of the proposed biased agonists MetI (purple), MetT (yellow), and NitroI (magenta) (upper panels). Conformational space explored by each ligand as a superposition of 1 every 20 frames per trajectory (middle panels). 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.

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_{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_{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.

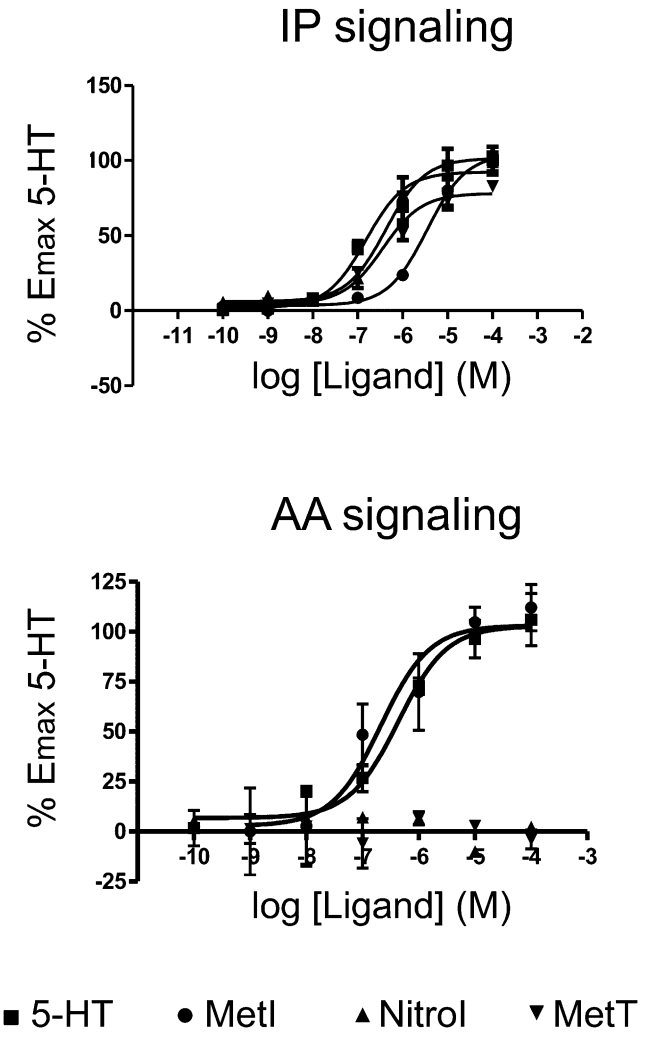

Fig. 3. Results from pharmacological characterization of novel compounds. Data correspond to the mean of three independent experiments with duplicate observations for each experiment.

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-HT$_{2A}$ 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-HT$_{2A}$ 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-HT$_{2A}$ 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

TABLE 1

Pharmacological data of novel compounds for the IP and AA pathways at human 5-HT$_{2A}$ receptors

Data correspond to mean ± S.D. EC$_{50}$ values (μM) of three independent experiments with duplicate observations. Dashed lines represent nonobservable efficacy values at the tested concentration range.

Ligand	EC$_{50}$ AA Signaling	E_{max} AA Signaling	EC$_{50}$ IP Signaling	E_{max} IP Signaling
Serotonin	0.433 ± 0.160	100 ± 6	0.122 ± 0.030	100 ± 3
MetI	0.210 ± 0.023	100 ± 6	3.520 ± 0.171	100 ± 5
MetT	—		0.437 ± 0.072	86 ± 2
NitroI	—		0.491 ± 0.051	104 ± 2

and S5.46 in the case of IP signaling. Based on this knowledge, we have discovered new biased ligands of unprecedented 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-HT_{2A} receptor that go beyond ligand-receptor interactions. Given that in our study we were only able to obtain compounds with very high levels of bias for IP signaling (MetT and NitroI), 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-HT_{2A} 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.

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