Molecular determinants for the interaction of the valvulopathic anorexigen norfenfluramine with the 5-HT$_{2B}$ receptor

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Running Title: Residues involved in norfenfluramine binding to 5-HT$_{2B}$ receptors

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Text pages: 25
Tables: 4
Figures: 8
References: 40
Words in Abstract: 249

Non-standard abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); GPCR, G protein-coupled receptor; VHD, valvular heart disease; PH, pulmonary hypertension; $E_{\text{max}}$, maximum relative efficacy
ABSTRACT

S-(+)-Norfenfluramine (SNF)—an active metabolite of the now-banned anorexigen fenfluramine—has been implicated in the drug’s appetite-suppressing actions and its life-threatening cardiovascular side-effects. SNF reduces appetite through serotonin 5-HT\textsubscript{2C} receptor activation; it causes cardiopulmonary toxicity through 5-HT\textsubscript{2B} receptor activation. Thus, we attempted to identify molecular determinants of SNF binding to 5-HT\textsubscript{2B} receptors distinct from those underlying SNF-5-HT\textsubscript{2C/2A} receptor interactions. Mutagenesis implicated V2.53 in SNF binding to 5-HT\textsubscript{2B} receptors. Ligand docking simulations suggested both V2.53 \(\gamma\)-methyl groups form stabilizing van der Waals’ (vdW) interactions with SNF’s \(\alpha\)-methyl group. A V2.53L mutation induced a 17-fold decrease in affinity, which molecular dynamics (MD) simulations suggested results from the loss of one 2.53-\(\alpha\)-methyl group vdW interaction. Supporting this, 1) the binding of NF analogs lacking an \(S\)-(+) \(\alpha\)-methyl group (RNF and \(\alpha\)-desmethyl-NF) was less sensitive to the V2.53L mutation, and 2) a V2.53A mutation decreased SNF affinity 190-fold, but RNF and \(\alpha\)-desmethyl-NF affinities only 16-fold and 45-fold, respectively. We next addressed whether the \(\alpha\)-methyl group of SNF contributes to 5-HT\textsubscript{2C/2A} receptor affinity. Removal of the \(\alpha\)-methyl group (\(\alpha\)-desmethyl-NF), which reduced 5-HT\textsubscript{2B} receptor binding three-fold compared to SNF, did not affect 5-HT\textsubscript{2C/2A} receptor binding. An \(\alpha\)-ethyl substituent (\(\alpha\)-ethyl-NF), which decreased 5-HT\textsubscript{2B} receptor affinity 46-fold, reduced 5-HT\textsubscript{2C} and 5-HT\textsubscript{2A} receptor binding 14-fold and five-fold, respectively. Finally, we determined that residue 2.53 affects SNF potency and efficacy at 5-HT\textsubscript{2B}, but not 5-HT\textsubscript{2C} and 5-HT\textsubscript{2A}, receptors. In conclusion, vdW interactions between residue 2.53 and the \(\alpha\)-methyl group of SNF contribute to the ligand’s 5-HT\textsubscript{2} receptor sub-type selective pharmacology.
INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic amine that regulates a broad spectrum of processes in both the central nervous system and in the periphery via no fewer than 15 plasma membrane receptors divided into seven families (Kroeze et al., 2002; Kroeze et al., 2003). All but one family belong to the Class A, rhodopsin-like, G protein-coupled receptor superfamily. The 5-HT₂ family receptors, comprising the 5-HT₂A, 5-HT₂B, and 5-HT₂C subtypes, represents one of the best-characterized groups of 5-HT receptors (Kroeze et al., 2002; Roth et al., 1998). These receptors are the major targets/sites of action of atypical antipsychotic medications (Meltzer, 1989; Roth et al., 2004), most hallucinogens (Glennon et al., 1984; Nichols, 2004; Roth et al., 2002), and some appetite suppressants (Moses and Wurtman, 1984; Neill and Cooper, 1989; Vickers et al., 2001).

The appetite suppressant fenfluramine, which is very effective in the treatment of obesity (Weintraub et al., 1992), was withdrawn from the US marketplace due to its association with valvular heart disease (VHD) (Connolly et al., 1997). Fenfluramine was also known to be associated with pulmonary hypertension (PH) (Brenot et al., 1993; Connolly et al., 1997; Pouwels et al., 1990; Simoneau et al., 1998). Fenfluramine-induced VHD and PH result from the activation of mitogenic 5-HT₂B receptors on heart valve and pulmonary artery interstitial cells (Fitzgerald et al., 2000; Launay et al., 2002; Rothman et al., 2000; Setola et al., 2003), leading to the formation of proliferative, fibromyxoid plaques that compromise tissue integrity and function (Steffee et al., 1999; Tomita and Zhao, 2002). Fenfluramine-induced anorexia, in contrast, appears to be mediated by activation of hypothalamic 5-HT₂C receptors (Heisler et al., 2002; Vickers et al., 2001). Thus, novel anorexigens that activate 5-HT₂C receptors and are devoid of 5-HT₂B receptor activity are expected to be safe and effective treatments for
obesity; the elucidation of 5-HT subB receptor-unique SNF-receptor intermolecular interactions could facilitate the rational design of such 5-HT subC receptor-selective agents.

In the present report, we identify residue 2.53 in 5-HT sub receptors as playing an important role in the sub-type selective in vitro pharmacology of S-(+)-norfenfluramine (SNF). Site-directed mutagenesis, SNF and NF congener binding studies, modeling, and ligand docking and MD simulations all suggest that both terminal methyl groups of V2.53 in the 5-HT subB receptor form stabilizing van der Waals’ (vdW) interactions with the α-methyl group of SNF. In addition, we provide functional data demonstrating that these interactions are also important for SNF-mediated activation of the 5-HT subB receptor. We also present evidence that the role of residue 2.53 on SNF binding to, and activation of, the 5-HT subB receptor is unique among 5-HT sub receptors.
MATERIALS AND METHODS

Receptor Constructs and Site-Directed Mutagenesis The human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ (INI editing isoform) receptors having been previously described (Rothman et al., 2000) were sub-cloned into the Not I site of pUniversal-Signal, a pIRES-neo (Clonetech)-based expression vector containing the following elements: a Kozak sequence followed by a cleavable membrane targeting sequence, followed by a FLAG epitope tag, all in frame with each other (Bhatnagar et al., 2004). Site-directed mutagenesis was performed using Stratagene’s QuikChange kit according to the manufacturer’s instructions. Mutagenized receptor cDNAs were sequenced by automated, PCR-based sequencing (Cleveland Genomics) to verify the introduction of the desired mutation and the absence of PCR-induced mutations.

Chemical Synthesis of Norfenfluramine (NF) Analogs α-Desmethyl-NF, or 2-[(3-trifluoromethyl)phenyl]ethylamine hydrochloride, was prepared by condensation of 3-(trifluoromethyl)benzaldehyde and nitromethane in the presence of ammonium acetate. The crude product was purified by Kugelrohr distillation ($90^\circ$C at 1 mmHg) to give a semisolid material. Although the nitrostyrene has been reported to be a solid (Menicagli and Samaritani, 1996), the product failed to crystallize. Reduction of the nitrostyrene intermediate with lithium aluminum hydride in dry tetrahydrofuran afforded a 47% yield of the target compound as its free base following Kugelrohr distillation. The hydrochloride salt was prepared and re-crystallized from 2-propanol (mp 100°C). Structure assignment was consistent with proton NMR data, and the hydrochloride salt analyzed (Atlantic Microlab, GA) to within 0.4% of theory for C, H, and N. Dengel et al. (1975) have previously prepared the free base of α-desmethyl-NF. Branch et al. (2002) have reported the synthesis of the hydrochloride salt (using a different route of synthesis) in the patent literature, but the salt was characterized only by low resolution mass spectrometry.
The α-ethyl homolog of NF has been mentioned only once before (Fuller et al., 1982), and this report described only the pharmacology, but not the synthesis or physicochemical characterization, of the agent. The α-ethyl homolog, 1-[(3-trifluoromethyl)phenyl]-2-aminobutane hydrochloride, was prepared by a method similar to that employed for the synthesis of α-desmethylNF. The condensation step used nitropropane in place of nitromethane to produce the nitrostyrene; the latter was converted to the oily free base of the target amine by lithium aluminum hydride reduction, purified by Kugelrohr distillation to obtain a 53% yield of the product, and converted to its hydrochloride salt (mp = 161-163°C following recrystallization from 2-propanol). The assigned structure was consistent with proton NMR data, and the salt analyzed (Atlantic Microlab, GA) to within 0.4% of theory for C, H, and N.

**Chemicals** Optically pure S-(+)- and R-(-)-NF were provided by Richard B. Rothman. LSD, pergolide, dihydroergotamine, serotonin (5-HT), and α-methyl-5-HT were all purchased from Sigma (St. Louis, MO).

**Cell Culture and Transfection** HEK-293T cells were maintained at 37°C, 5% CO₂ in 75-cm flasks (Costar) with Dulbecco’s Modified Eagle Medium (Gibco; Gaithersburg, MD) containing 100 mM sodium pyruvate (Gibco; Gaithersburg, MD), 100 U/ml penicillin (Gibco; Gaithersburg, MD), 100 µg/ml streptomycin (Gibco; Gaithersburg, MD) (DMEM) and 10% fetal bovine serum (Gibco; Gaithersburg, MD) (DMEM, 10% FBS). At confluence, cells were trypsinized, harvested, and split 1:5 into four 10-cm plates containing 10 ml DMEM, 10% FBS (for transfection) and one 75-cm flask containing 15 ml DMEM, 10% FBS (for cell maintenance). One day after seeding into 10-cm dishes (Costar) (i.e. at ~90% confluence), the medium was removed and replaced with 10 ml OptiMEM (Gibco; Gaithersburg, MD). Each 10-cm plate was transfected with 24 µg of receptor plasmid using Lipofectamine 2000 (Gibco; Gaithersburg, MD) exactly as specified by the manufacturer. Twenty-four hrs after transfection, cells
were processed for either radioligand binding assays or inositol phosphate accumulation assays as described below.

**Radioligand Binding Assays** Twenty-four hrs after transfection, the medium was removed and replaced with DMEM containing 5% dialyzed FBS (Gibco; Gaithersburg, MD). After 24 hrs, the cells were incubated overnight in serum-free DMEM. The next day, the cells were harvested by scraping, pelleted, and resuspended in lysis buffer (50 mM Tris-HCl, pH 6.9). The membranes were then pelleted by centrifugation and, after removal of the supernatant, the membrane fraction was frozen at -80°C (if not used immediately). Radioligand binding assays were set up in 24 wells of a 96-well plate (1 ml/well capacity) as follows: 25 µl of 10 µM [³H]LSD (PerkinElmer; Wellesley, MA), 25 µl of membrane pellet that had been resuspended in 700 µl ice-cold binding buffer (50 mM Tris-HCl, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.4), 25 µl vehicle (binding buffer) or 10X test compound dissolved in binding buffer (i.e., SNF, RNF, α-desmethyl-NF, α-R, S-(±)-ethyl-NF, 5-HT, α-methyl-5-HT, dihydroergotamine, or pergolide) at various concentrations spanning seven orders of magnitude (2 wells/concentration), and 200 µl binding buffer, such that the final [³H]LSD and test compound concentrations were 1X. Reactions were equilibrated in the dark for 1 hr at room temperature (~22°C), and then receptor-ligand complexes were harvested by vacuum filtration onto Whatman (Florham Park, NJ) GF/C filters (pre-soaked in 0.3% polyethyleneimine) then washed three times with ice-cold 50 mM Tris-HCl, pH 6.9 using a Brandel 24-well harvester. The filters were dried overnight, and each was added to a 6-ml vial, into which 4 ml EcoScint liquid scintillation cocktail (National Diagnostics; Atlanta, GA) was then added. The total [³H]LSD binding present on each filter was quantified by liquid scintillation counting using a Wallac (Wellesley, MA) LSC. The log $K_D$ (for LSD) or log $K_i$ (for all other compounds) and $B_{\text{max}}$ values were determined using Prism 4.0 (GraphPad; San Diego, CA) by fitting the average total [³H]LSD binding (in DPM) from several independent experiments, plotted as a function of the log [compound], to a
homologous (for LSD) or heterologous (for all other compounds) competition model that takes into account ligand depletion (GraphPad; San Diego, CA) and shares the log $K_D$ or log $K_i$ among all data sets.

**Inositol Phosphate Accumulation Assays** Twenty-four hrs after transfection, cells in 10-cm plates were harvested by trypsinization and resuspended in 25 ml DMEM, 5% dialyzed FBS and seeded (1 ml cell suspension/well) in a 24-well, poly-L-lysine-coated plate. The next day, the medium was removed and replaced with BME (Gibco; Gaithersburg, MD) containing 1 µCi/ml [³H]myo-inositol (PerkinElmer; Wellesley, MA) (500 µl/well). Twenty-four hours later, the medium was replaced (1 ml/well) with 1X Hank’s Balanced Salt Solution (Gibco; Gaithersburg, MD) containing 25 mM sodium bicarbonate, 11 mM glucose, and 10 mM LiCl (IP buffer). Agonists (5-HT, SNF, and RNF) were diluted in IP buffer to 100X and added (10 µl/well) to the cells. After a 1-hr incubation at 37°C, the buffer was removed and replaced with 1 ml of 1 mM formic acid to extract the cytosolic fraction. The [³H]inositol phosphates accumulated in the cytosol were isolated by loading the cytosolic fraction onto Dowex anion exchange resin (formate form) columns, washing the columns twice with water (first with 2 ml/column, then with 10 ml/column), then washing with 10 ml wash buffer (5 mM sodium borate, 50 mM sodium formate)/column, and finally eluting with 10 ml elution buffer (100 mM formic acid, 200 mM sodium formate)/column into 30-ml vials containing 10 ml/vial 3a70B liquid scintillation cocktail (Research Products International). [³H]inositol phosphate accumulation in each sample was quantified by liquid scintillation counting using a Wallac LSC. The log $EC_{50}$ and $E_{max}$ values were determined using Prism 4.0 (GraphPad) by fitting the average total [³H]inositol phosphate from several independent experiments, plotted as a function of the log [agonist], to a 3-parameter logistic equation (sigmoidal concentration-response) and sharing the log $EC_{50}$ and $E_{max}$ among all data sets.

**Modeling, Ligand Docking Simulations, and Molecular Dynamics (MD) Simulations** Three-dimensional models of the human 5-HT$_{2B}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors were constructed using our
rhodopsin-based (Palczewski et al., 2000), refined, validated 3-D homology model of the rat 5-HT$_{2A}$R (Shapiro et al., 2002) as a template. The raw sequence of each receptor was first fit onto the template using the DeepView (Glaxo-Smithkline; Research Triangle Park, NC) modeling program’s Magic Fit function, then up-loaded to the Swiss Model server (http://swissmodel.expasy.org//SWISS-MODEL.html). The returned PDB files were loaded into Sybyl 6.91 modeling and MD software (Tripos, Inc.; St. Louis, MO) and, after atom typing and calculation of charges, energy minimized using the Powell method under an AMBER7 FF99 force field with an 8-Å non-bonded cut-off and a distance-dependent dielectric, constant = 4. The local minimum was identified using a Wolfe line search for successive iterations that differed by less than 0.05 kcal/mol·Å. Ten such models were generated and the structures averaged, resulting in an apo receptor model. To generate a reference structure for ligand docking simulations, 5-HT was manually placed in the interhelical space of the 5-HT$_{2B}$ receptor model according to three conserved features of 5-HT binding the receptor: 1) the protonated amine was 2-3 Å away from D3.32, and 2) the 5’-OH of the ligand was 2-3 Å away from S3.36, and 3) the aromatic moiety was near the “aromatic box” delineated by W3.28, W6.48, F6.51, and F6.52 (Manivet et al., 2002; Roth et al., 1997). To open up the binding pocket, the receptor side chains within 6 Å of the manually-docked 5-HT were energy minimized as above. The interatomic distances in the resulting structure were in good agreement with previous descriptions of biogenic amine ligand binding to 5-HT$_{2}$-family receptors (Kristiansen et al., 2000; Manivet et al., 2002; Roth et al., 1997; Shapiro et al., 2000). The ligand was then extracted from the minimized receptor, which was used as the receptor description file (RDF) for the simulated docking of SNF and RNF by the FLEXX module of Sybyl6.91 (Tripos, Inc.; St. Louis, MO). The ligand binding site was defined as those residues 6 Å within D3.32. Only those solutions that were consistent with the conserved features described above were retained for consideration.
To explore the possible effects of a mutation on SNF or NF analog binding, the mutation was computationally introduced into selected ligand docking simulation solutions and ten rounds of computer-simulated annealing (CSA) were performed to explore possible side chain orientations relative to the bound ligand. Docked solutions bearing the mutation to be studied were heated to and held at 700 K for 1 ps, then cooled (0.5 K/fs) to 200 K during a 1-ps interval using an exponential temperature-vs-time ramping method. Snapshots were taken every 5 fs. During the MD simulations, the distance between the protonated amine group of the ligand and the nearest carboxylate oxygen of D3.32, the ligand atoms, and the $\alpha$-carbon backbone of the receptor, and all atoms greater than 8 Å away from the mutated side chain were constrained. For MD calculations during CSA, an AMBER7 FF99 force field was applied with an 8-Å non-bonded cut-off and a distance-dependent dielectric, constant = 4 using a non-bonded cut-off. The resulting structure from each round of CSA was examined vis-à-vis the orientation of the mutated residue’s side chain relative to the ligand.
RESULTS

Effect of Point Mutations on Ligand Affinity In order to identify residues involved in the sub-type selective binding of the anorexigen S-(+)-norfenfluramine (SNF) to 5-HT\textsubscript{2} family receptors, we constructed every possible 5-HT\textsubscript{2B}-to-5-HT\textsubscript{2C/2A} receptor mutant residing near a putative ligand binding pocket. To do so, we first generated a molecular model of the human 5-HT\textsubscript{2B} receptor by homology model building using our bovine rhodopsin-based (Palczewski et al., 2000), refined, validated 3-D homology model of the rat 5-HT\textsubscript{2A} receptor (Shapiro et al., 2002) as a template. To verify that our rhodopsin-based model was similar in predicted structure to the published co-ordinates of bovine rhodopsin (Palczewski et al., 2000), we superimposed our 5-HT\textsubscript{2B} receptor homology model onto 1HZXA (the bovine rhodopsin PDB file). All absolutely conserved transmembrane helix residues aligned and were oriented very similarly relative to each other (data not shown). We next identified putative ligand binding residues in the 5-HT\textsubscript{2B} receptor (i.e., those residues in the extracellular half of the transmembrane helices having their side chains oriented into the interhelical space) that were not conserved in linear alignments of the 5-HT\textsubscript{2} receptor transmembrane helices (Figure 1). We then measured the affinity constant ($K_i$) of SNF at wild type and point mutant 5-HT\textsubscript{2B} receptors in which one non-conserved, putative ligand binding residue was mutated to its 5-HT\textsubscript{2C} and/or 5-HT\textsubscript{2A} receptor analog. As shown in Figure 2A and Table 1, only the V2.53L and the M5.39V mutations caused a decrease in SNF affinity: the V2.53L (5-HT\textsubscript{2B/2C}-to-5-HT\textsubscript{2A}) mutation decreased SNF affinity 17-fold, while the M5.39V (5-HT\textsubscript{2B}-to-5-HT\textsubscript{2C/2A}) mutation led to a tenfold decrease in ligand affinity. The two mutations in tandem decreased SNF affinity 37-fold (Table 1). Because V2.53 was predicted by our model to reside in the putative binding pocket, we chose to investigate further its potential, direct role in the 5-HT\textsubscript{2B} receptor-selective binding of SNF.
In the 5-HT\(^2\text{C}\) receptor, residue 2.53 is a valine. However, the V2.53L mutation in the 5-HT\(^2\text{C}\) receptor only caused a nine-fold decrease in SNF affinity (i.e., half as large a decrease as that caused by the mutation in the 5-HT\(^2\text{B}\) receptor) (Figure 2B and Table 1). The inverse L2.53V mutation in the 5-HT\(^2\text{A}\) receptor had no effect on SNF affinity (Figure 2C and Table 1). Thus, the role of residue 2.53 in SNF binding to 5-HT\(^2\) receptors is sub-type selective, being most important in the 5-HT\(^2\text{B}\) receptor, of moderate importance in the 5-HT\(^2\text{C}\) receptor, and not at all important in the 5-HT\(^2\text{A}\) receptor. This mirrors the rank order potency of SNF at the 5-HT\(^2\) family receptors (i.e., \(K_i = 22\text{ nM}, 170\text{ nM}, \) and 1,900 nM, respectively).

To ensure that the apparent decrease in SNF affinity due to the V2.53L mutation did not result from altered affinity for the radioligand ([\(^3\text{H}\)]LSD), we measured the \(K_i\) of LSD at wild type and V2.53L 5-HT\(^2\text{B}\) receptors. As shown in Figure 3 (A,B) and Table 2, the V2.53L mutation had little effect on LSD affinity. Indeed, none of the mutations studied herein altered LSD affinity by more than two-fold (Figure 3, Table 2, and data not shown), suggesting that none of the mutations dramatically alters receptor folding/topology. We also examined the effect of the V2.53L mutation on four other 5-HT\(^2\text{B}\) receptor agonists: 5-HT, \(\alpha\)-methyl-5-HT, dihydroergotamine, and pergolide (Figure 3A,B and Table 2). The V2.53L mutation had less or no effect on the binding of the other agonists assayed, the largest effect being a five-fold decrease in affinity for dihydroergotamine (Figure 3B and Table 2). The preceding observations demonstrate that the V2.53L mutation markedly and uniquely affects SNF binding to 5-HT\(^2\text{B}\) receptors.

**Modeling, Ligand Docking Simulations, and MD Simulations of Ligand Binding to 5-HT\(^2\text{B}\) Receptors** To investigate the possible atomic interactions by which V2.53 in the 5-HT\(^2\text{B}\) receptor contributes to high-affinity SNF binding, we performed ligand docking simulations. Each of the 30 solutions generated was inspected to determine whether 1) the protonated amine nitrogen of the ligand
was close enough to D3.32 to form the conserved, anchoring ionic interaction, and 2) whether the aromatic group of the ligand was near one or more of the conserved aromatic residues known to stabilize biogenic amine ligand binding (Choudhary et al., 1993; Manivet et al., 2002; Roth et al., 1997). Only 16 of the 30 docked structures met the first criterion \( (\text{i.e., the protonated amine nitrogen of the ligand was less than 3.0 Å away from one of the carboxylate oxygens of D3.32}) \). Of these, only two placed the ligand close enough to V2.53 for the latter to have an affect on SNF affinity, as was indicated by our mutant receptor binding data (Figure 4A-D). The lower energy solution (solution 1, total score = -10.58 kcal/mol) placed the \( \alpha \)-methyl group carbon of SNF 3.73 Å and 3.76 Å away from the terminal \( \gamma \)-methyl group carbons of V2.53 (Figure 4A,B). The other solution (solution 2, total score = -10.48 kcal/mol) placed the \( \alpha \)-methyl group carbon of SNF 3.68 Å and 4.57 Å away from the terminal \( \gamma \)-methyl group carbons of V2.53 (Figure 4C,D).

To determine which of the two solutions was best able to explain our mutagenesis data, we computationally introduced the V2.53L mutation into each solution, and then did ten rounds of computer-simulated annealing to examine several possible orientations of the V2.53L side chain relative to the ligand (Figure 4E-H). For the solution 1-based MD simulations, the average distances ± SEM between the terminal \( \delta \)-methyl group carbons of V2.53L and the \( \alpha \)-methyl group carbon of SNF in the 10 lowest energy structures were 3.35 ± 0.05 Å and 4.6 ± 0.1 Å (Figure 4E,F). We compared the V2.53L terminal \( \delta \)-methyl group-ligand \( \alpha \)-methyl group intercarbon distances to those between the terminal \( \gamma \)-methyl group carbons of V2.53 and the \( \alpha \)-methyl group carbon of SNF in solution 1 \( (\text{i.e., 3.73 Å and 3.76 Å}) \). Assuming favorable vdW’ (vdW) methyl group-methyl group interactions occur at intercarbon distances of 3-4 Å, our MD simulations suggested that the net effect of the V2.53L mutation resulted from the loss of a stabilizing vdW interaction between the 2.53 side chain and the \( \alpha \)-methyl group of the ligand. For solution 2-based MD simulations, the V2.53L terminal \( \delta \)-methyl group-ligand \( \alpha \)-methyl group intercarbon
distances ± SEM were 3.57 ± 0.05 Å and 4.71 ± 0.09 Å (Figure 4G,H). Comparing these receptor side-
chain-ligand intercarbon distances to those between the terminal γ-methyl group carbons of V2.53 and the α-methyl group carbon of SNF in solution 2 (3.58 Å and 4.57 Å) revealed very little difference. Accordingly, solution 2 did not predict a change in the number of stabilizing vdW interactions between the 2.53 side chain and the α-methyl group of SNF upon mutation of V2.53 to leucine. Thus, vis-à-vis the V2.53L mutation, solution 1 best modeled our experimental observations.

As for M5.39, in none of the 30 solutions was the residue predicted to be close enough to SNF to directly influence ligand binding. Furthermore, in none of the CSA simulations was the M5.39 side chain predicted to be within less than 4 Å of bound SNF. Nevertheless, our data demonstrate a role for M5.39 in SNF binding, which our current model suggests is indirect.

To further test the predictive power of our model, we performed identical MD simulations and analyses after introducing a conservative V2.53I mutation into solution 1 (Figure 5). The average distances ± SEM between the terminal γ- and δ-methyl group carbons of V2.53I and the α-methyl group carbon of SNF in the ten lowest energy structures were 3.91 ± 0.05 Å and 3.8 ± 0.1 Å, respectively. Comparing these values to those for the terminal γ-methyl group carbons of V2.53 and the α-methyl group carbons of SNF in solution 1 (3.73 Å and 3.76 Å), revealed very little difference. Thus, solution 1 predicted that the conservative V2.53I mutation would not alter vdW interactions with the α-methyl group of SNF. Based on these predictions, we made the V2.53I mutant receptor and measured its affinity for SNF, which we expected to be similar to that of the wild type receptor. As predicted by our model, the affinity of the V2.53I mutant receptor for SNF was not altered by the mutation (Table 1). Solution 1, therefore, accurately modeled the effects of both the V2.53L and the V2.53I mutations. Interestingly, the V2.53I mutation did decrease 5-HT and α-methyl-5-HT affinity by approximately ten-fold, while the
affinity for DHE, pergolide, and LSD (the radioligand) were affected less than three-fold by the mutation (Figure 3C and Table 2).

Assuming that the R enantiomer of norfenfluramine (RNF) binds in the same orientation as SNF, our model suggested that the α-H of RNF would appose V2.53 and that the α-methyl group would project ‘down’ into the interhelical space, away from V2.53. Because hydrogen atoms, due to their smaller atomic radius, are less efficient than bulkier methyl groups at forming attractive vdW, and because the α-H of RNF would be more than 4 Å away from V2.53, our model predicted that RNF would bind to the wild type 5-HT2B receptor with a lower affinity than SNF. As shown in Table 3, RNF displayed a three-fold decrease in affinity compared to SNF at the 5-HT2B receptor. Furthermore, because the R-(-) α-methyl group of RNF would be more than 4 Å away from V2.53 and, therefore not likely to form stabilizing vdW interactions, our model predicted that the V2.53L mutation would have a smaller effect on RNF affinity than it did on SNF affinity. As shown in Table 3, the affinity for RNF was decreased only three-fold by the V2.53L mutation, compared to a 17-fold decrease in SNF affinity due to the mutation.

We also measured the affinity of the wild type and V2.53L mutant 5-HT2B receptors for α-desmethyl-NF. As shown in Table 3, α-desmethyl-NF, like RNF, exhibited a three-fold reduction in affinity for the 5-HT2B receptor compared to SNF. The affinity for α-desmethyl-NF was reduced five-fold by the V2.53L mutation (Table 3)—a reduction very similar to that observed for RNF, suggesting that the decrease in RNF affinity due to the V2.53L mutation did not result from altered interactions with the α-methyl group. The affinity of the NF analogs lacking an S-(+) α-methyl group (RNF and α-desmethyl-NF) was very similar to that of SNF at the V2.53L 5-HT2B receptor, suggesting a lack of productive interactions between the V2.53L side chain and the α-methyl group of SNF. Our model, however, suggested that the V2.53L side chain forms one vdW interaction with the α-methyl group of SNF. As such, we would have expected the V2.53L 5-HT2B receptor to have lower affinity for α-desmethyl-NF than for SNF. To
investigate potential interactions between the V2.53L and α-desmethyl-NF, we replaced the α-methyl group of SNF with a hydrogen atom in our V2.53L 5-HT2B receptor model, then performed computer-simulated annealing to explore several possible orientations of the V2.53L side chain relative to α-desmethyl-NF. The results of our MD simulations predicted that in the absence of an S-(+) α-methyl group, at least one of the terminal δ-methyl groups of V2.53L was close enough to the α-carbon of α-desmethyl-NF to form a stabilizing vdW interaction (Figure 6A,B). With SNF docked, however, the V2.53L side chain was predicted to be oriented such that only one of the terminal δ-methyl groups apposed the ligand—an orientation that accommodates the S-(+) α-methyl group (Figure 4A,B). Thus, our MD simulations provided a plausible explanation for the lack of difference in SNF and α-desmethyl-NF affinity at the V2.53L 5-HT2B receptor: removal of the S-(+) methyl group, with which one of the V2.53L terminal δ-methyl groups forms a stabilizing vdW interaction, allows V2.53L to project into the binding pocket, permitting a stabilizing vdW interaction between a V2.53L terminal δ-methyl group and the α-carbon of α-desmethyl-NF.

Given our mutagenesis data and modeling predicting that the α-methyl group of SNF is within 4 Å of V2.53, we hypothesized that an α-carbon substituent bulkier than a methyl group (i.e., an ethyl group) would reduce NF binding to the wild type 5-HT2B receptor. As shown in Table 3, α-R,S-(±)-ethyl-NF exhibited a 46-fold lower affinity for the wild type 5-HT2B receptor compared to SNF. According to our model, the reduction in affinity due to the α-ethyl substitution was most likely due to 1) steric hindrance between the α-ethyl group of the ligand and V2.53, or 2) the adoption of a different binding orientation due to non-tolerance of the α-ethyl group by V2.53. In either case, our results with α-R,S-(±)-ethyl-NF corroborated our model implicating interactions between the α-methyl group of SNF and the γ-methyl groups of V2.53 in the ligand’s high-affinity binding to 5-HT2B receptors. At the V2.53L 5-HT2B receptor, α-R,S-(±)-ethyl-NF affinity was disrupted less than two-fold compared to SNF. Initially, because leucine
is bulkier than valine, we had predicted the V2.53L mutant 5-HT$_{2B}$ receptor to be more sensitive to the bulkier $\alpha$-carbon substituent of $\alpha$-$R,S$(±)-ethyl-NF. To explore possible rationales for the lack of differences between SNF and $\alpha$-$R,S$(±)-ethyl-NF affinity at the V2.53L 5-HT$_{2B}$ receptor, we replaced the $\alpha$-methyl group of SNF with an ethyl group in our model, and then performed computer-simulated annealing to explore several possible orientations of the V2.53L side chain relative to the $\alpha$-ethyl group. As depicted in Figure 6C,D our MD simulations predicted the V2.53L side chain and the $\alpha$-ethyl group of the ligand to be oriented such that one V2.53L terminal $\delta$-methyl group could form a favorable vdW interaction with the $\alpha$-carbon-proximal methylene group of the $\alpha$-ethyl substituent; the terminal methyl group of the $\alpha$-ethyl substituent projected ‘down’ into the binding pocket, away from the V2.53L side chain. Thus, our MD simulations suggested that for SNF, $\alpha$-desmethyl-NF, and $\alpha$-$R,S$(±)-ethyl-NF binding to the V2.53L 5-HT$_{2B}$ receptor, the V2.53L side chain forms one stabilizing vdW interaction with each, consistent with the compounds having similar affinity (Table 3).

If, indeed, the decrease in SNF affinity in the V2.53L mutant was due to the loss of one stabilizing vdW interaction between the 2.53 side chain and the $\alpha$-methyl group of SNF—and not steric hindrance due to the bulkiness of the V2.53L side chain—we expected that a V2.53A mutation would result in the loss of two stabilizing vdW interactions and, thus, an even larger decrease in SNF affinity than that caused by the V2.53L mutation. To test this prediction, we made the V2.53A mutant receptor and measured its affinity for SNF. As shown in Figure 7 and Table 1, and as predicted by our model, the V2.53A mutation caused a dramatic 190-fold decrease in SNF affinity. The affinities of other agonists were altered much less by the V2.53A mutation (Table 2), suggesting a SNF-specific effect. These observations strongly suggest that the effect of the V2.53L mutation on SNF affinity was most likely due to a decrease in vdW stabilization rather than steric hindrance.
Having established the importance of interactions between V2.53 and the \( \alpha \)-methyl group of SNF in ligand binding to 5-HT\(_{2B} \) receptors, we sought to determine whether the \( \alpha \)-methyl group of SNF contributes to the ligand’s binding to 5-HT\(_{2C} \) and 5-HT\(_{2A} \) receptors. To do so, we measured the affinity of the SNF analogs RNF, \( \alpha \)-desmethyl-NF and \( \alpha \)-\( R,S \)-(\( \pm \))-ethyl-NF at the two receptors. As shown in Table 3, the affinities of \( \alpha \)-desmethyl-NF at the 5-HT\(_{2C} \) and 5-HT\(_{2A} \) receptors were not different than SNF and RNF affinities, suggesting that the \( \alpha \)-methyl group of SNF does not contribute significantly to ligand binding to those receptors. In contrast, NF analogs lacking an \( S \)-(+) \( \alpha \)-methyl group (i.e., RNF and \( \alpha \)-desmethyl-NF) exhibited a three-to-four-fold reduction in binding affinity at wild type 5-HT\(_{2B} \) receptors, reflecting productive receptor-\( S \)-(+) \( \alpha \)-methyl group interactions. The affinities of the 5-HT\(_{2C} \) and 5-HT\(_{2A} \) receptors for \( \alpha \)-\( R,S \)-(\( \pm \))-ethyl-NF were reduced 14-fold and five-fold, respectively, compared to SNF; the wild type 5-HT\(_{2B} \) receptor exhibited a dramatic 46-fold decrease in affinity for the compound compared to SNF. Thus, a bulkier \( \alpha \)-carbon substituent had the most dramatic effect (~50-fold) on NF binding to 5-HT\(_{2B} \) receptors, a much lesser (~ten-fold) effect on NF binding to 5-HT\(_{2C} \) receptors, and a modest (five-fold) effect on NF binding to 5-HT\(_{2A} \) receptors.

**Effect of Point Mutations on Agonist Potency and Efficacy**

For each of the wild type and point mutant 5-HT\(_{2B} \) receptors, we also measured SNF potency (EC\(_{50} \)) and relative efficacy compared to the full agonist 5-HT (\( E_{\text{max}} \)) (Figure 8 and Table 4). As was true for ligand affinity, agonist potency was decreased only by the V2.53L and M5.39V mutations (Figure 8A-C and Table 4). In contrast to its effect on SNF affinity at 5-HT\(_{2B} \) receptors, the V2.53L mutation in the 5-HT\(_{2C} \) receptor did not alter the agonist’s potency (Table 4). This is in line with our finding that SNF affinity was less sensitive to the V2.53L mutation in the 5-HT\(_{2C} \) receptor than in the 5-HT\(_{2B} \) receptor. The L2.53V mutation in the 5-HT\(_{2A} \) receptor caused a dramatic 20-fold decrease in the potency of SNF (Table 4)—a change in the opposite direction than would have been predicted based on a role for residue 2.53 in contributing to the rank order
potency of SNF at 5-HT\textsubscript{2} receptors. Only the V2.53L mutation in the 5-HT\textsubscript{2B} receptor altered the agonist relative efficacy: SNF efficacy was increased 36\% by the mutation (Figure 7A,B and Table 5). In terms of RNF potency, the V2.53L mutation in both the 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C} receptors increased potency to a similar extent: approximately 2-fold (Figure 8A,B and Table 4). The L2.53V mutation in the 5-HT\textsubscript{2A} receptor decreased RNF potency 30-fold (Table 4), an effect similar to that observed for SNF potency. With respect to RNF relative efficacy, as with SNF relative efficacy, only the V2.53L mutation in the 5-HT\textsubscript{2B} receptor affected the parameter, decreasing it a dramatic 65\% (Figure 8A,B and Table 4). However, while without effect on agonist efficacy itself, the M5.39V mutation in tandem with the V2.53L mutation eliminated the latter mutation’s effect on SNF efficacy and increased its effect on RNF efficacy \~1.2-fold (Figure 8E and Table 4).
DISCUSSION

The main finding reported herein is that V2.53 in the 5-HT\textsubscript{2B} receptor contributes to the high-affinity binding of SNF through vdW interactions between both V2.53 terminal $\gamma$-methyl groups and the $\alpha$-methyl group of the ligand. Of the 11 5-HT\textsubscript{2B}-to-5-HT\textsubscript{2C/2A} mutations tested, only the V2.53L (5-HT\textsubscript{2B/2C}-to-5-HT\textsubscript{2A}) and M5.39V (5-HT\textsubscript{2B}-to-5-HT\textsubscript{2C/2A}) mutations resulted in substantial changes in SNF affinity (Figure 2 and Table 1). Residue 2.53 in the 5-HT\textsubscript{2C} receptor is also a valine; however, the V2.53L mutation in the 5-HT\textsubscript{2C} receptor diminished SNF binding only half as much as it did in the 5-HT\textsubscript{2B} receptor (Figure 2 and Table 1). The inverse L2.53V mutation in the 5-HT\textsubscript{2A} receptor did not affect SNF affinity (Figure 2 and Table 1), suggesting that interactions between V2.53 and SNF are more important in 5-HT\textsubscript{2B} receptors than in 5-HT\textsubscript{2C} receptors, and that L2.53 plays no role in SNF binding to the 5-HT\textsubscript{2A} receptor. Given the rank order affinity of SNF for 5-HT\textsubscript{2} receptors (5-HT\textsubscript{2B}>5-HT\textsubscript{2C}>5-HT\textsubscript{2A}), our data suggest that interactions between V2.53 in the 5-HT\textsubscript{2B} receptor and the $\alpha$-methyl group of the ligand contribute to sub-type selectivity.

To ascertain whether the V2.53L mutation affected the binding of other agonists, we measured the affinity of two tryptamines (5-HT and $\alpha$-methyl-5-HT) and three ergolines (dihydroergotamine, pergolide, and LSD) at 5-HT\textsubscript{2B} receptors. The data presented in Table 2 demonstrate that 2.53 mutations in the 5-HT\textsubscript{2B} receptor did not globally perturb agonist binding. Notably, LSD (i.e., the radioligand) affinity was altered less than two-fold. Taken together, the preceding results demonstrate that the effect of the V2.53L mutation is selective for SNF.

To explore how V2.53 might contribute to SNF affinity at 5-HT\textsubscript{2B} receptors, we performed ligand docking simulations. Of the 30 solutions generated, only two were both consistent with conserved features of biogenic amine ligand binding and predicted SNF to be close enough to V2.53 for the residue
to directly affect binding (i.e. within 4 Å) (Figure 4). In both of these, the α-methyl group of the ligand was predicted to be near both (solution 1) or one (solution 2) of the V2.53 terminal γ-methyl groups. After introduction of the V2.53L mutation into each solution and computer-simulated annealing to explore possible side chain orientations relative to the α-methyl group of SNF, both solutions predicted only one vdw interaction (Figure 4). For solution 2, then, V2.53 and V2.53L were predicted to interact similarly with the ligand, each contributing one stabilizing vdw interaction. For solution 1, V2.53 was predicted to contribute two stabilizing vdw interactions and V2.53L was predicted to contribute only one. The energetic cost (i.e., the ΔΔG calculated from the data in Table 1) of the V2.53L mutation on SNF binding was +1.66 kcal/mol. Others have measured the contribution of methyl group-methyl group interactions to ligand binding energy, with values ranging from +0.4 kcal/mol to +1.4 kcal/mol (Bigler et al., 1993; Faergeman et al., 1996; Huang et al., 1995; Kawaguchi and Kuramitsu, 1998; Lu et al., 1997; Morton et al., 1995; Oue et al., 1997). The value calculated from our data (+1.66 kcal/mol) is thus consistent with the V2.53L mutation causing the loss of a 2.53 methyl group-SNF α-methyl group vdw interaction, a prediction of our modeling and MD simulations. In addition, the ΔΔG for the V2.53A mutation reveals a +2.94-kcal/mol difference in SNF binding energy compared to the wild type. Thus, removal of the second stabilizing methyl group-methyl group vdw interaction costs +2.94 kcal/mol – (+1.66 kcal/mol) = +1.28 kcal/mol, again consistent with reported interaction energies and further supporting our model predicting two stabilizing vdw interactions between V2.53 and the α-methyl group of SNF.

To further test our modeling and MD simulations, we studied the effect of a V2.53I mutation on 2.53-SNF interactions. Computer-simulated annealing predicted that V2.53I would, like V2.53, form two stabilizing vdw interactions with the α-methyl group of SNF (Figure 5). Thus, we predicted that the mutation would not affect SNF affinity. As shown in Table 1, the V2.53I 5-HT_{2B} receptor’s affinity for SNF was not significantly different from that of the wild type, in agreement with our modeling and MD
simulations. This result validates our computational approach and substantiates the prediction that the \( \alpha \)-methyl group of SNF interacts with both terminal methyl groups of V2.53.

Given the apparent importance of the \( \alpha \)-methyl group-V2.53 interaction for SNF binding to 5-HT\(_2B\) receptors, we measured the affinity of the wild type and V2.53L mutant receptors for NF analogs bearing \( \alpha \)-carbon ‘mutations.’ Assuming that RNF binds in the same orientation as SNF, our model predicts that the \( \alpha \)-methyl group of RNF will project ‘down’ towards the cytoplasmic face of the receptor and the \( \alpha \)-H will appose V2.53. We reasoned that both the increased distance of the \( \alpha \)-methyl group from V2.53 and the smaller vdW radius of the apposing \( \alpha \)-H would render RNF much less sensitive to the V2.53L mutation. As shown in Table 3, RNF affinity was indeed decreased only three-fold by the V2.53L mutation, compared to a 17-fold effect on SNF affinity. Similarly, \( \alpha \)-desmethyl-NF also exhibited only a three-fold decreased affinity at V2.53L 5-HT\(_2B\) receptors compared to its affinity at the wild type receptor (Table 3). Thus, the 5-HT\(_2B\) receptor-selective effect of the V2.53L mutation on NF binding requires the ligand to bear an \( \alpha \)-methyl group in the S\((\pm)\) configuration; the R\((-)\) \( \alpha \)-methyl group does not appear to contribute to NF binding to the wild type 5-HT\(_2B\) receptor—an observation that is consistent with the similar affinities of \( \alpha \)-desmethyl-NF, which lacks an \( \alpha \)-methyl group, and RNF. We also measured the affinity of \( \alpha \)-R,S\((\pm)\)-ethyl-NF for wild type 5-HT\(_2B\) receptors. We hypothesized that, if the \( \alpha \)-methyl group of SNF is within 4 Å of either terminal \( \gamma \)-methyl group of V2.53, an \( \alpha \)-ethyl substituent would be poorly tolerated due to steric hindrance. As shown in Table 3, the 5-HT\(_2B\) receptor exhibited a 46-fold decreased affinity for \( \alpha \)-R,S\((\pm)\)-ethyl-NF compared to SNF, further supporting our model wherein V2.53 of the 5-HT\(_2B\) receptor is within 4 Å of the \( \alpha \)-methyl group of SNF—close enough for both terminal \( \gamma \)-methyl groups to form stabilizing vdW interactions with the \( \alpha \)-methyl group of ligand. In addition, the 5-HT\(_2C\) and 5-HT\(_2A\) receptors displayed considerably smaller reductions in \( \alpha \)-R,S\((\pm)\)-ethyl-NF affinity—14-fold and five-fold, respectively (Table 3). One possible explanation for the reduced sensitivity of the 5-
HT$_{2C}$ and 5-HT$_{2A}$ receptors to $\alpha$-carbon ‘mutations’ in the ligands is that the ligands may bind in a different orientation to the 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors than does SNF to the 5-HT$_{2B}$ receptor.

Our studies of NF congener binding to the V2.53L 5-HT$_{2B}$ are also consistent with interactions between V2.53 and the $\alpha$-methyl group of SNF. For instance, our modeling and MD simulations predicted the formation of one stabilizing vdW interaction between V2.53L and SNF (compared to two such interactions between V2.53 and the $\alpha$-methyl group of SNF). At first glance, one would have expected ‘removal’ of the $\alpha$-methyl group (i.e., RNF or $\alpha$-desmethyl-NF) to eliminate the remaining stabilizing vdW interaction, thereby decreasing affinity. However, we observed that RNF and $\alpha$-desmethyl-NF bound to the V2.53L 5-HT$_{2B}$ receptor with affinities similar to that of SNF. In accord with the previous observations, our modeling and MD simulations suggested that, absent an $S$-$(\pm)$ $\alpha$-methyl group, both V2.53L terminal $\delta$-methyl groups can project into the binding pocket and form at least one stabilizing vdW interaction with the $\alpha$-carbon of $\alpha$-desmethyl-NF. Thus, our MD simulations provided a plausible molecular rationale for the observation that SNF, RNF and $\alpha$-desmethyl-NF displayed similar affinities at the V2.53L 5-HT$_{2B}$ receptor. Our modeling and MD simulations also provided an explanation for the similar affinities of $\alpha$-$R,S$-$(\pm)$-ethyl-NF and SNF for the V2.53L 5-HT$_{2B}$ receptor. With $\alpha$-$R,S$-$(\pm)$-ethyl-NF bound in the pocket, our MD simulations predicted that the V2.53L side chain orients such that one of the V2.53L terminal $\delta$-methyl groups can form a stabilizing vdW interaction; the other terminal $\delta$-methyl group projects away from the ligand, leaving room for the bulkier $\alpha$-carbon substituent.

A much simpler explanation for the effect of the V2.53L mutation on SNF binding to the 5-HT$_{2B}$ receptor is that the mutation results in steric hindrance with some group(s) on the ligand. However, the observations that 1) the bulky V2.53I side chain did not affect SNF binding, and 2) generating a larger region of bulk tolerance via a V2.53A mutation resulted in a much larger perturbation of SNF binding than did the V2.53L mutation (i.e., 46-fold vs. 17-fold) argue against the simpler explanation.
the fact that the wild type 5-HT\textsubscript{2B} receptor displays smaller decreases in affinity for RNF and α-desmethyl-NF than that caused by V2.53L mutation on SNF affinity suggests the existence of additional factors.

A secondary finding of this study is that M5.39 also participates in SNF binding. We attempted to explain the role of M5.39 in SNF binding, as we had for V2.53, using modeling, ligand docking simulations, and MD simulations. In none of the computer-simulated annealing results, however, was M5.39 predicted to be less than 10 Å away from bound SNF. A possible explanation for our M5.39 data that is consistent with our model is that the residue stabilizes, through hydrophobic and/or hydrogen bonding interactions, extracellular loop 2 (e2) in the binding pocket in such a way that the loop favorably interacts with bound SNF. Indeed, the analogous dopamine D2 receptor residue (V5.39) along with e2 likely plays a role in the binding of some ligands (Shi and Javitch, 2004). Alternatively, given its position at the extracellular surface of the receptor, M5.39 might participate in initial receptor-ligand interactions that guide SNF ‘down’ into the binding pocket.

We also determined SNF potency and efficacy at wild type and mutant 5-HT\textsubscript{2} receptors (Figure 8 and Table 4). SNF potency, like its binding affinity, was only decreased at V2.53L and M5.39V mutant 5-HT\textsubscript{2B} receptors (Figure 8 and Table 4). As for agonist relative efficacy, a unique role for V2.53 in 5-HT\textsubscript{2B} receptor activation became apparent (Figure 8 and Table 5). That is, both SNF and RNF $E_{\text{max}}$ were altered only in the 5-HT\textsubscript{2B} receptor, and only by the V2.53L—and not the V2.53I—mutation.

In conclusion, we have discovered that residue 2.53—a valine—plays a role in SNF binding to, and activation of, the 5-HT\textsubscript{2B} receptor that is unique among 5-HT\textsubscript{2} family receptors. Our studies suggest the existence of two stabilizing vdW’ interactions between the terminal methyl groups of V2.53 and the α-methyl group of SNF in the ligand-bound receptor. These interactions also have consequences on receptor function, since SNF potency and efficacy are both affected when these interactions are perturbed. Thus,
vdW interactions between V2.53 and the α-methyl group of SNF contribute to the sub-type selective pharmacology. Targeting these interactions via ligand ‘mutations’ (e.g., by synthesizing SNF analogs lacking, or bearing bulkier, α-carbon substituents in the S-(+) orientation) may result in a more selective 5-HT2C agonist. Indeed, as we show herein, NF congeners either lacking an α-methyl group or bearing a bulkier α-ethyl substituent exhibit reductions in 5-HT2B—but not 5-HT2C or 5-HT2A—receptor affinity. Similar ligand-receptor interactions that are unique to the 5-HT2B receptor among 5-HT2 receptors are likely to exist for SNF-induced activation as well, and a better understanding of these could guide drug design efforts towards an effective appetite suppressant devoid of VHD- and PH-inducing potential.
ACKNOWLEDGEMENTS

We are grateful to Richard B. Rothman at the National Institute on Drug Abuse and the NIMH Chemical Synthesis Program for providing optically-pure SNF and RNF, and to David Larson, David Derrington, William Curtis, and Michael Dolan at Tripos, Inc. for invaluable, expert assistance with the Sybyl 6.91 software. We also thank Wesley Kroeze for assistance with statistical analyses and for the critical reading of our manuscript, and Douglas Sheffler for help with image preparation.
REFERENCES


FOOTNOTES

This work was supported by an American Heart Association pre-doctoral fellowship to VS and NIH RO1MH57635, KO2MH01366, and National Institutes of Mental Health Psychoactive Drug Screening Program to BLR.

Case Western Reserve University School of Medicine, Departments of ¹Biochemistry, ³Psychiatry, and ³Neurosciences; ²Virginia Commonwealth University, School of Pharmacy, Department of Medicinal Chemistry
Figure 1. 3-D molecular model of the human 5-HT$_{2B}$ receptor showing putative ligand binding residues that are non-conserved among 5-HT$_2$ family receptors. Side chains shown by mutagenesis and radioligand binding assays to be involved in SNF binding are labeled in green; those shown not to affect SNF binding are labeled in white. Atom color code: carbon (white); nitrogen (blue); oxygen (red); hydrogen (cyan); sulfur (yellow). The α-carbons backbone of the seven helices is shown in purple. (See Materials and Methods for details regarding generation of the model.)

Figure 2. Representative competition binding isotherms for SNF at wild type and mutant 5-HT$_2$ receptors. The percent total binding of ~1 nM [³H]LSD remaining in the presence of the indicated concentration of SNF is shown for WT, V2.53L, and M5.39V 5-HT$_{2B}$ receptors (A); WT and V2.53L 5-HT$_{2C}$ receptors (B); WT and L2.53V 5-HT$_{2A}$ receptors (C); WT and V2.53I 5-HT$_{2B}$ receptors (D). Data are presented as the mean ± SE of at least three independent experiments measured in duplicate. To obtain log $K_i$ values from these data, the data were fit to a heterologous competition model of radioligand binding to one class of receptor sites that takes ligand depletion into account (GraphPad Prism 4.0). Non-specific radioligand binding never exceeded 20% of the total radioligand binding.

Figure 3. Competition binding isotherms for several 5-HT$_{2B}$R agonist ligands at wild type and V2.53L 5-HT$_{2B}$ receptors. The percent total binding of ~1 nM [³H]LSD remaining in the presence of 5-HT (A), α-methyl-5-HT (B), pergolide (C), and dihydroergotamine (DHE, E) is indicated for WT and V2.53L 5-HT$_{2B}$ receptors. Data are presented as the mean ± SE of at least three independent experiments measured in duplicate. To obtain log $K_i$ values from these data, the data were fit to a heterologous competition model of radioligand binding to one class of receptor sites that takes ligand depletion into account (GraphPad Prism 4.0). Non-specific radioligand binding never exceeded 20% of the total binding.
radioligand binding. The V2.53A and V2.53I 5-HT\textsubscript{2B} receptors were assayed and analyzed simultaneously (see Table 2).

**Figure 4A. 3-D molecular models showing the results of ligand docking simulations that are consistent with conserved features of biogenic amine ligand binding.** Simulated docking of SNF to the model shown in Figure 3 was performed using the FLEXX module of Sybyl 6.91 software. The solutions consistent with conserved features of biogenic amine ligand binding are shown. A (solution 1), C (solution 2): Predicted orientation of SNF relative to conserved residues important for biogenic amine ligand binding. B (solution 1), D (solution 2): Space fill representation of predicted docking of SNF relative to V2.53, in which the terminal methyl groups of V2.53 and the \(\alpha\)-methyl group of SNF are indicated by arrowheads and an arrow, respectively. Atom color code: carbon (white); nitrogen (blue); oxygen (red); hydrogen (cyan); sulfur (yellow). The \(\alpha\)-carbons backbone of the seven helices is shown in purple.

**Figure 4B. Representative energy-minimized structures from ten rounds of computer-simulated annealing of solution 1 (A,B) and solution 2 (C,D) after insertion of the V2.53L mutation.** The results predict, in both cases, one stabilizing van der Waals’ interaction between the \(\alpha\)-methyl group of SNF (arrows) and the terminal \(\delta\)-methyl groups of V2.53L (arrowheads). Atom color code: carbon (white); nitrogen (blue); oxygen (red); hydrogen (cyan); sulfur (yellow). The \(\alpha\)-carbons backbone of the seven helices is shown in purple.

**Figure 5. Representative energy-minimized structure from ten rounds of computer-simulated annealing of solution 1 after insertion of the V2.53I mutation.** The result predicts two stabilizing van
der Waals’ interaction between the α-methyl group of SNF (arrow) and the terminal methyl groups of V2.53I (arrowheads). (A) Stick representation; (B) space fill representation. Atom color code: carbon (white); nitrogen (blue); oxygen (red); hydrogen (cyan); sulfur (yellow). The α-carbons backbone of the seven helices is shown in purple.

Figure 6. Representative energy-minimized structure from ten rounds of computer-simulated annealing of solution 1 bearing the V2.53L after addition or removal of SNF α-carbon substituents. (A,B) The result predicts a stabilizing van der Waals’ interaction between a terminal δ-methyl group of V2.53L (arrowheads) and the α-carbon of α-desmethy-NF (arrow). (C,D) The result predicts a stabilizing van der Waals’ interaction between a terminal δ-methyl group of V2.53L (arrowheads) and the α-carbon-proximal methylene group of the α-ethyl substituent of α-ethyl-NF; the terminal ethyl group of the α-ethyl substituent points ‘down’ away from V2.53L. Atom color code: carbon (white); nitrogen (blue); oxygen (red); hydrogen (cyan); sulfur (yellow). The α-carbons backbone of the seven helices is shown in purple.

Figure 7. Competition binding isotherms for SNF at V2.53A 5-HT2B receptors. The percent total binding of ~1 nM [3H]LSD remaining in the presence of the indicated concentration of SNF is shown. Data are presented as the mean ± SE of at least three independent experiments measured in duplicate. To obtain log $K_i$ values from these data, the data were fit to a heterologous competition model of radioligand binding to one class of receptor sites that takes ligand depletion into account (GraphPad Prism 4.0). Non-specific radioligand binding was never exceeded 20% of the total radioligand binding. SNF congeners (RNF, α-desmethyl-NF, α-R,S-(±)-NF) were assayed and analyzed in parallel (see Table 3).
Figure 8. **Concentration-response isotherms for agonist-stimulated inositol phosphate accumulation.** Agonist-, concentration-dependent activation of WT (A), V2.53L (B), M5.39V (C), V2.53I (D), and V2.53L,M5.39V (E) 5-HT$_{2B}$ receptors transiently expressed in HEK293 cells is shown. Data represent the mean ± SE of at least three independent experiments measured in duplicate. Average baseline and maximal drug-stimulated [$^3$H]IP accumulation (in DPM) in wild type 5-HT$_{2B}$ receptor-expressing cells were: 2,700 ± 400 (baseline), 13,000 ± 3,000 (5-HT), 10,000 ± 2,000 (SNF), and 11,000 ± 2,000 (RNF); corresponding values for V2.53L 5-HT$_{2B}$ receptor-expressing cells were: 900 ± 100 (baseline), 13,000 ± 5,000 (5-HT), 14,000 ± 5,000 (SNF), and 4,000 ± 2,000. To obtain log EC$_{50}$ and E$_{\text{max}}$ values from concentration-response data, the data were fit to a three parameter logistic concentration-response model (GraphPad Prism 4.0). E$_{\text{max}}$ is expressed as percent of the maximum response to 5-HT.
Table 1. Affinity constants ($K_i$'s) for SNF binding to wild type and mutant 5-HT$_2$ receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$pK_i$ S-NF ± SE $(K_i$ S-NF, nM)</th>
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<tbody>
<tr>
<td>5-HT$_{2B}$ WT</td>
<td>7.66 ± 0.02 (22)</td>
</tr>
<tr>
<td>A1.35P</td>
<td>7.81 ± 0.07 (15)</td>
</tr>
<tr>
<td>A1.35S</td>
<td>7.77 ± 0.07 (17)</td>
</tr>
<tr>
<td>L1.38S</td>
<td>7.76 ± 0.06 (17)</td>
</tr>
<tr>
<td>I1.39T</td>
<td>7.84 ± 0.07 (14)</td>
</tr>
<tr>
<td>V1.42I</td>
<td>7.82 ± 0.09 (15)</td>
</tr>
<tr>
<td>V2.53L</td>
<td>6.43 ± 0.04 (370)</td>
</tr>
<tr>
<td>V2.53I</td>
<td>7.45 ± 0.04 (35)</td>
</tr>
<tr>
<td>V2.53A</td>
<td>5.48 ± 0.08 (3,300)</td>
</tr>
<tr>
<td>L3.29I</td>
<td>7.65 ± 0.07 (22)</td>
</tr>
<tr>
<td>M5.39V</td>
<td>6.65 ± 0.06 (220)</td>
</tr>
<tr>
<td>V2.53L, M5.39V</td>
<td>6.09 ± 0.08 (810)</td>
</tr>
<tr>
<td>E7.36N</td>
<td>7.80 ± 0.06 (16)</td>
</tr>
<tr>
<td>S7.45C</td>
<td>7.65 ± 0.05 (22)</td>
</tr>
<tr>
<td>5-HT$_{2A}$ WT</td>
<td>5.73 ± 0.04 (1900)</td>
</tr>
<tr>
<td>5 HT$_{2A}$ L2.53V</td>
<td>5.74 ± 0.04 (1800)</td>
</tr>
<tr>
<td>5-HT$_{2C}$ WT</td>
<td>6.77 ± 0.05 (170)</td>
</tr>
<tr>
<td>5-HT$_{2C}$ V2.53L</td>
<td>5.8 ± 0.1 (2000)</td>
</tr>
</tbody>
</table>

$p$ values were obtained from F-tests comparing curve fits of competition binding isotherms for two receptors: $^a p<0.05$ compared the appropriate wild type 5-HT$_2$ receptor; $^b p<0.05$ compared to the V2.53L 5-HT$_{2B}$ receptor; $^c p<0.05$ compared to the M5.39V 5-HT$_{2B}$ receptor.
Table 2. Affinity constants ($K_i$'s) for other 5-HT$_2B$ receptor agonist ligands at wild type and V2.53 mutant 5-HT$_2B$ receptors.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug</th>
<th>WT $pK_i$ ($K_i$, nM)</th>
<th>V2.53L $pK_i$ ($K_i$, nM)</th>
<th>V2.53A $pK_i$ ($K_i$, nM)</th>
<th>V2.53I $pK_i$ ($K_i$, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>7.3 ± 0.1</td>
<td>7.17 ± 0.08</td>
<td>6.5 ± 0.2*</td>
<td>6.2 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(50)</td>
<td>(68)</td>
<td>(300)</td>
<td>(600)</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-methyl-5-HT</td>
<td>7.8 ± 0.1</td>
<td>7.3 ± 0.1*</td>
<td>6.4 ± 0.2*</td>
<td>6.7 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td>(50)</td>
<td>(400)</td>
<td>(200)</td>
<td></td>
</tr>
<tr>
<td>DHE</td>
<td>7.63 ± 0.08</td>
<td>6.9 ± 0.2*</td>
<td>7.3 ± 0.2</td>
<td>7.2 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td>(120)</td>
<td>(50)</td>
<td>(60)</td>
<td></td>
</tr>
<tr>
<td>Pergolide</td>
<td>7.68 ± 0.09</td>
<td>7.5 ± 0.1</td>
<td>6.6 ± 0.1*</td>
<td>7.3 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td>(30)</td>
<td>(200)</td>
<td>(50)</td>
<td></td>
</tr>
<tr>
<td>LSD</td>
<td>8.59 ± 0.05</td>
<td>8.34 ± 0.04*</td>
<td>8.41 ± 0.05</td>
<td>8.61 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.6)</td>
<td>(4.6)</td>
<td>(3.9)</td>
<td>(2.4)</td>
<td></td>
</tr>
</tbody>
</table>

* $p<0.05$ from F-tests comparing curve fits of competition binding isotherms for wild type and mutant 5-HT$_{2B}$R. Average $B_{max}$ values in DPM, fit directly and adjusted for radioligand specific activity and protein content, were as follows: WT, 3.7 ± 0.7 pmol/mg; V2.53L, 9 ± 2 pmol/mg; V2.53A, 7 ± 1 pmol/mg; V2.53I, 2.4 ± 0.2 pmol/mg.
Table 3. Affinity constants ($K_i$'s) for SNF and congeners at wild type and V2.53L 5-HT$_{2B}$ receptors and wild type 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors.

<table>
<thead>
<tr>
<th>Drug</th>
<th>WT p$K_i$ ($K_i$, nM)</th>
<th>V2.53L p$K_i$ ($K_i$, nM)</th>
<th>WT2C p$K_i$ ($K_i$, nM)</th>
<th>WT2A p$K_i$ ($K_i$, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNF</td>
<td>7.76 ± 0.05 (17)</td>
<td>6.64 ± 0.07$^d$ (230)</td>
<td>7.16 ± 0.07 (69)</td>
<td>5.74 ± 0.08 (1,800)</td>
</tr>
<tr>
<td>RNF</td>
<td>7.21 ± 0.04 (62)</td>
<td>6.84 ± 0.07$^d$ (140)</td>
<td>7.07 ± 0.08 (85)</td>
<td>5.85 ± 0.07 (1,400)</td>
</tr>
<tr>
<td>α-desmethyl-NF</td>
<td>7.26 ± 0.06$^a$ (55)</td>
<td>6.58 ± 0.05$^b,d$ (260)</td>
<td>7.06 ± 0.07 (87)</td>
<td>5.87 ± 0.06 (1,300)</td>
</tr>
<tr>
<td>α-ethyl-NF</td>
<td>6.11 ± 0.08$^{a,b,c}$ (780)</td>
<td>6.44 ± 0.08$^{a,b,c,d}$ (360)</td>
<td>6.0 ± 0.1 (1,000)$^{a,b,c}$</td>
<td>5.0 ± 0.1$^{a,b,c}$ (10,000)</td>
</tr>
</tbody>
</table>

$p$ values were obtained from F-tests comparing curve fits of competition binding isotherms for two receptors: $^a$p<0.05 compared to S-(+)-NF; $^b$p<0.05 compared to R-(-)-NF; $^c$p<0.05 compared to α-desmethyl-NF; $^d$p<0.05 compared to WT.
Table 4. Potency (EC\(_{50}\)) and relative efficacy (\(E_{\text{max}}\)) values for SNF and RNF at wild type and point mutant 5-HT\(_2\) receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pEC(<em>{50}) S-NF ± SE (EC(</em>{50}) S-NF, nM)</th>
<th>pEC(<em>{50}) R-NF ± SE (EC(</em>{50}) R-NF, nM)</th>
<th>SNF (E_{\text{max}}) ± SE (% 5-HT max.)</th>
<th>RNF (E_{\text{max}}) ± SE (% 5-HT max.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT(_2) WT</td>
<td>7.66 ± 0.07 (22)</td>
<td>6.62 ± 0.07 (240)</td>
<td>75 ± 3</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>A1.35P</td>
<td>7.7 ± 0.1 (20)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1.35S</td>
<td>7.69 ± 0.06 (20)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L1.38S</td>
<td>7.51 ± 0.08 (30)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>I1.39T</td>
<td>7.50 ± 0.09 (31)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>V1.42I</td>
<td>7.44 ± 0.08 (36)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>V2.53L</td>
<td>7.09 ± 0.07(^a) (82)</td>
<td>6.99 ± 0.07(^a) (100)</td>
<td>101.7 ± 0.6(^a)</td>
<td>28.2 ± 0.5(^a)</td>
</tr>
<tr>
<td>V2.53I</td>
<td>7.7 ± 0.1 (20)</td>
<td>7.4 ± 0.1(^a) (40)</td>
<td>87 ± 4(^a)</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>L3.29I</td>
<td>7.92 ± 0.03(^a) (12)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M5.39V</td>
<td>7.19 ± 0.09(^a) (64)</td>
<td>6.7 ± 0.1 (200)</td>
<td>70 ± 3</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>V2.53L, M5.39V</td>
<td>6.49 ± 0.05(^a, b, c) (320)</td>
<td>6.80 ± 0.08(^b) (160)</td>
<td>73 ± 4(^b)</td>
<td>18 ± 1(^a, b, c)</td>
</tr>
<tr>
<td>E7.36N</td>
<td>7.6 ± 0.1 (20)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S7.45C</td>
<td>7.8 ± 0.2 (10)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5-HT(_2)A WT</td>
<td>6.7 ± 0.2 (200)</td>
<td>6.6 ± 0.2 (300)</td>
<td>75 ± 3</td>
<td>69 ± 5</td>
</tr>
<tr>
<td>5HT(_2)A L2.53V</td>
<td>5.3 ± 0.1(^a) (5000)</td>
<td>5.07 ± 0.09(^a) (8500)</td>
<td>70 ± 3</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>5-HT(_2)C WT</td>
<td>7.2 ± 0.2 (70)</td>
<td>6.7 ± 0.1 (200)</td>
<td>93 ± 6</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>5-HT(_2)C V2.53L</td>
<td>7.2 ± 0.1 (70)</td>
<td>6.97 ± 0.8(^a) (100)</td>
<td>85 ± 5</td>
<td>82 ± 4</td>
</tr>
</tbody>
</table>

\(E_{\text{max}}\) is reported as % maximum response to 5-HT. \(p\) values were obtained from F-tests comparing curve fits of concentration-response isotherms for two receptors: \(^a\)\(p<0.05\) compared to the appropriate WT 5-HT\(_2\) receptor; \(^b\)\(p<0.05\) compared to V2.53L 5-HT\(_2\) WT receptor; \(^c\)\(p<0.05\) compared to the M5.39V 5-HT\(_2\)B receptor. ND = not determined.