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Downregulation of 5-HT$_7$ Serotonin Receptors by the Atypical Antipsychotics Clozapine and Olanzapine. Role of Motifs in the C-Terminal Domain and Interaction with GASP-1

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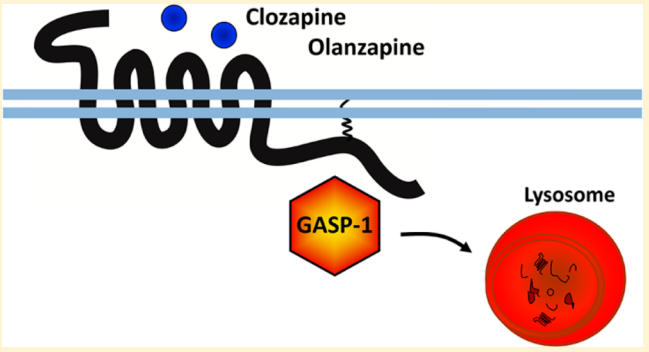
ABSTRACT: The human 5-HT$_7$ serotonin receptor, a G-protein-coupled receptor (GPCR), activates adenylyl cyclase constitutively and upon agonist activation. Biased ligands differentially activate 5-HT$_7$ serotonin receptor desensitization, internalization and degradation in addition to G protein activation. We have previously found that the atypical antipsychotics clozapine and olanzapine inhibited G protein activation and, surprisingly, induced both internalization and lysosomal degradation of 5-HT$_7$ receptors. Here, we aimed to determine the mechanism of clozapine- and olanzapine-mediated degradation of 5-HT$_7$ receptors. In the C-terminus of the 5-HT$_7$ receptor, we identified two YXXΦ motifs, LR residues, and a palmitoylated cysteine anchor as potential sites involved in receptor trafficking to lysosomes followed by receptor degradation. Mutating either of these sites inhibited clozapine- and olanzapine-mediated degradation of 5-HT$_7$ receptors and also interfered with G protein activation. In addition, we tested whether receptor degradation was mediated by the GPCR-associated sorting protein-1 (GASP-1). We show that GASP-1 binds the 5-HT$_7$ receptor and regulates the clozapine-mediated degradation. Mutations of the identified motifs and residues, located in or close to Helix-VIII of the 5-HT$_7$ receptor, modified antipsychotic-stimulated binding of proteins (such as GASP-1), possibly by altering the flexibility of Helix-VIII, and also interfered with G protein activation. Taken together, our data demonstrate that binding of clozapine or olanzapine to the 5-HT$_7$ receptor leads to antagonist-mediated lysosomal degradation by exposing key residues in the C-terminal tail that interact with GASP-1.



KEYWORDS: *5-HT$_7$ receptor, clozapine, olanzapine, GASP-1, lysosome, Helix-VIII*

G-protein-coupled receptor (GPCR)-mediated signaling is a highly regulated process where ligand-stimulated GPCRs undergo rapid desensitization and internalization into endosomes, subsequently followed by either recycling back to the plasma membrane or a degradative pathway.[1−3] Such postendocytic sorting of internalized receptors is dependent on the primary structure of each receptor, particularly the intracellular C-terminal tail and the particular ligand bound to the receptor. While arrestins and G-protein-coupled receptor kinases are involved in desensitization and internalization of GPCRs,[4,5] less is known about the proteins involved in the postendocytotic sorting mechanism of GPCRs. Few proteins have been identified to target GPCRs to a degradation pathway.[6] One family of such proteins is the GPCR-associated sorting protein (GASP) family, that interacts with the C-terminal tail of a large number of GPCRs[7] and mediates lysosomal sorting and thereby degradation of certain receptors, such as δ opioid, D$_2$ and D$_3$ dopamine, CB$_1$ cannabinoid, GPCR55, and US28 receptors.[8−16] A common trait among receptors interacting with GASP-1 is two residues located

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within Helix-VIII and alanine substitution of these residues inhibited GASP-1 interaction with the C-terminal tail of the δ-opioid and β_1-adrenergic receptors. These residues, which seem conserved among a subset of GPCRs interacting with GASP-1 may play a critical role in the interaction with GASP-1.[17]

The human 5-HT$_7$ receptor is a GPCR coupled to G$_s$, that activates adenylyl cyclase (AC) constitutively and upon agonist activation.[18,19] It is expressed as three different splice variants (a, b, and d) which differ only in the carboxy-terminus[20−22] and does not recruit β-arrestin upon activation, despite displaying internalization.[23] The 5-HT$_7$ receptor is expressed in both the central nervous system (CNS) and in peripheral tissues. Most atypical and some typical antipsychotic drugs bind to 5-HT$_7$ receptors with high affinity[24] and the 5-HT$_7$ receptor has been suggested to be involved in the treatment of schizophrenia and depression.[22,25,26]

Numerous studies have shown that different ligands, both agonists and antagonists/inverse agonists, can stabilize distinct GPCR conformations[27,28] leading to differential effects.[29−35] This phenomenon is termed functional selectivity or biased agonism.[36] We have previously demonstrated the presence of functional selectivity at 5-HT$_7$ receptors.[23,37] Of particular interest is the finding that the atypical antipsychotics clozapine and olanzapine, in addition to displaying full inverse agonism at 5-HT$_7$ receptors with respect to activation of adenylyl cyclase,[18,38] mediate internalization and targeting of the receptors to lysosomes for degradation, while the endogenous agonist (5-HT) and other ligands only mediate internalization without receptor degradation.[23] The downregulation was differentially regulated across the three 5-HT$_7$ splice variants, where the shortest 5-HT$_{7(b)}$ receptor variant was most prone to downregulation.[23] 5-HT and a different subset of inverse agonists (SB269970 and methiothepin), on the other hand, induced both homo- and heterologous desensitization without downregulation of 5-HT$_7$ receptors.[37] Taken together, this indicates that clozapine and olanzapine stabilize distinct receptor conformations that are responsible for lysosomal degradation of 5-HT$_7$ receptors. Some of these conformations might expose key residues of the receptor responsible for lysosomal trafficking.

Previous studies of several GPCR C-terminal tails (including the 5-HT$_7$ receptor C-terminal tail) have suggested that specific residues in Helix-VIII are important for interaction with GASP-1.[17,39] In the present study, we identified, within or in proximity of Helix-VIII of 5-HT$_7$ receptors, the two residues commonly suggested for GASP-1 interaction (L391/R392), two YXXΦ motifs (Y395/L398, Y403/I406) and the palmitoylated cysteine-anchor (C401) as potential sites involved in clozapine- and olanzapine-mediated sorting of 5-HT$_7$ receptors to lysosomes.

RESULTS

Clozapine- and Olanzapine-Mediated Downregulation Is Reversed by the Deletion of the C-Terminal Tail of 5-HT$_{7(b)}$ Receptors. We have previously shown that HEK293 cells stably expressing 5-HT$_{7(a,\ b\ and\ d)}$ receptors undergo a slow clozapine- and olanzapine-mediated receptor downregulation through sorting of receptors to lysosomes. There were differences among the splice variants, as the 5-HT$_{7(b)}$ receptor splice variant was significantly more downregulated.[23,37] Here, we show that in QBI-HEK293 cells transiently expressing 5-HT$_{7(b)}$ receptors, 24 h incubation with clozapine or olanzapine reduced 5-HT$_{7(b)}$ receptor densities to 55 ± 6% and 54 ± 6% of control, respectively (Figure 1). Removing the C-terminal tail of the 5-HT$_7$ receptor (ΔC-tail),

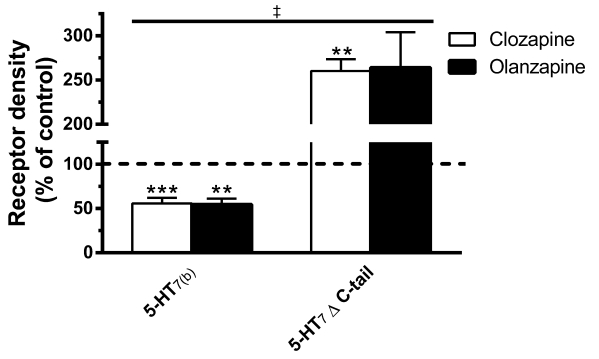

Figure 1. Incubation with clozapine or olanzapine induces downregulation of 5-HT$_{7(b)}$ receptors and up-regulation of ΔC-tail receptors. Receptor density (B_{max}) in membranes of QBI-HEK293 cells expressing the 5-HT$_{7(b)}$ and 5-HT$_7$ ΔC-tail receptors preincubated for 24 h with either 1 μM clozapine or 20 μM olanzapine presented as a percent of control (sister plates of cells incubated without drug). Radioligand binding was performed with increasing concentrations of [^{3}H]5-CT in the absence (total binding) and presence (nonspecific binding) of 10 μM 5-HT for 1 h at 24 °C. Total receptor levels (B_{max}) and binding affinity (K_d) were determined as described in Methods. B_{max} and K_d of untreated and treated cells for 5-HT$_{7(b)}$ and 5-HT$_7$ΔC-tail receptors are reported in Table 1. B_{max} of the 5-HT$_7$ΔC-tail receptors was 22 ± 8% of WT. The data shown are mean ± SEM of 4−10 experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared to nontreated cells (control) (one-way ANOVA with Bonferroni's multiple comparison test); ‡p < 0.01 compared to WT receptor incubated with clozapine or olanzapine (two-way ANOVA with Bonferroni's multiple comparison test).

not only prevented clozapine- and olanzapine-mediated receptor degradation, but significantly increased the density of 5-HT$_7$ ΔC-tail receptors to 260 ± 13% and 264 ± 40% of control, respectively. The affinities of [^{3}H]5-CT for the 5-HT$_{7(b)}$ and 5-HT$_7$ ΔC-tail receptors were not significantly modified after 24 h incubation with clozapine or olanzapine (Table 1). This suggests that the C-terminal tail of the 5-HT$_{7(b)}$ receptor is involved in sorting of receptors to lysosomes.

5-HT$_{7(b)}$ Receptors Bind GASP-1. GASP-1 has been shown to bind to the C-terminus of many GPCRs and is responsible for postendocytotic sorting of receptors to lysosomes for degradation.[8−16] GASP-1 has also been shown to bind to a GST-tagged C-terminus of the 5-HT$_{7(a)}$ receptor, which is only 13 amino acids longer than the 5-HT$_{7(b)}$ receptor.[17] In order to verify the interaction between GASP-1 and the 5-HT$_{7(b)}$ receptor, we performed a coimmunoprecipitation assay between the 5-HT$_{7(b)}$ receptor and GFP-GASP-1 or a GFP-tagged C-terminal domain of GASP-1 (cGASP-1; previously demonstrated to be responsible for the interaction between GASP-1 and several GPCRs[9−11]). As shown in Figure 2, both GFP-GASP-1 and GFP-cGASP-1 coimmunoprecipitated with the 5-HT$_{7(b)}$ receptor, demonstrating that GASP-1 binds to 5-HT$_{7(b)}$ receptors and that this interaction is adequately mediated by the C-terminus of GASP-1.

GASP-1 Regulates Clozapine-Mediated Downregulation of 5-HT$_{7(b)}$ Receptors. In order to determine the role of GASP-1-binding to 5-HT$_{7(b)}$ receptors, we overexpressed cGASP-1, which previously was shown to inhibit binding between GASP-1 and GPCRs and thus prevent ligand-mediated downregulation.[9,13] As shown in Figure 3, overexpression of

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Table 1. Affinity and Receptor Density of the 5-HT$_{7(b)}$ Receptor and 5-HT$_{7(b)}$ Mutant Receptors without and with Ligand Preincubation[a]

FLAG-tagged 5-HT$_{7(b)}$ receptor	K_d (nM)			B_{max} (pmol/mg protein)
	no drug	clozapine	olanzapine	
WT	0.39 ± 0.06	0.42 ± 0.15	0.35 ± 0.09	4.32 ± 0.80
ΔC-tail[d]	0.24 ± 0.03	0.41 ± 0.09	0.42 ± 0.13	0.69 ± 0.34[c]
L391A/R392A	0.44 ± 0.07	0.37 ± 0.07	0.36 ± 0.05	1.05 ± 0.21[c]
Y395A/L398A	0.40 ± 0.09	0.47 ± 0.12	0.51 ± 0.13	1.92 ± 0.18[b]
C401S	0.54 ± 0.13	0.32 ± 0.12	0.09 ± 0.01	1.40 ± 0.39[c]
Y403A/I406A	0.48 ± 0.13	0.63 ± 0.13	0.61 ± 0.26	2.06 ± 0.20[b]
Y395A/L398A + Y403A/I406A	0.60 ± 0.17	0.45 ± 0.05	0.65 ± 0.39	1.34 ± 0.20[c]

[a]Affinity (K_d) and receptor density (B_{max} in pmol/mg protein) of [^{3}H]5-CT-binding was determined in membranes of QBI-HEK293 cells transiently transfected with the indicated 5-HT$_{7(b)}$ variant receptors. K_d was measured in membranes expressing the FLAG-tagged WT, ΔC-tail, L391A/R392A, Y395A/L398A, C401S, Y403A/I406A, and Y395A/L398A + Y403A/I406A 5-HT$_{7(b)}$ receptors preincubated 24 h with either vehicle (no drug), 1 μM clozapine, or 20 μM olanzapine. Membranes were incubated with increasing concentrations of [^{3}H]5-CT in the absence (total binding) or presence (nonspecific binding) of 10 μM 5-HT. K_d and B_{max} were determined, and data were analyzed as described in Methods. The data shown are mean ± SEM from 3 to 17 independent experiments. [b]$p < 0.01$. [c]$p < 0.001$ versus WT (one-way ANOVA with Bonferroni's multiple comparison test). [d]The 5-HT$_7$ ΔC-tail receptor is Cmyc-tagged.

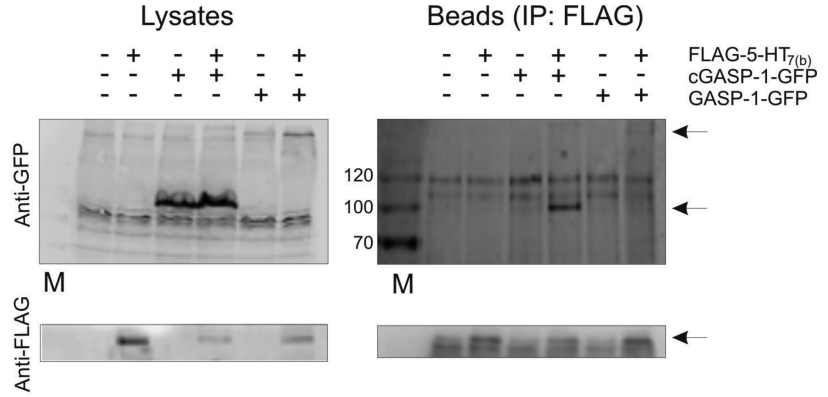

Figure 2. The C-terminal tail of GASP-1 interacts with 5-HT$_{7(b)}$ receptors. HEK293T cells were transiently transfected with the indicated plasmids. 48 h post-transfection cells were lysed and 5% of the lysates were subjected to SDS-PAGE followed by immunoblotting with anti-GFP and anti-FLAG. The rest of the lysates were subjected to immunoprecipitation with anti-FLAG. Direct interaction between the 5-HT$_{7(b)}$ and GASP-1 or cGASP-1 were detected by immunoblotting with anti-FLAG and anti-GFP. Representative immunoblots from three similar experiments are shown.

cGASP-1 significantly attenuated clozapine-mediated receptor downregulation compared to pcDNA3.1-transfected cells. In the absence of cGASP-1, clozapine decreased 5-HT$_{7(b)}$ receptor densities by 51 ± 6%, whereas in cells overexpressing cGASP-1, clozapine only reduced the receptor density by 13 ± 10%. The affinities (K_d values) of [^{3}H]5-CT for untreated cells and clozapine-treated HEK293 cells stably expressing 5-HT$_{7(b)}$ receptors were 1.26 ± 0.58 and 1.05 ± 0.26 nM, respectively, while cells transiently overexpressing cGASP-1 displayed K_d values of 0.92 ± 0.31 and 0.96 ± 0.09 nM, respectively, indicating that the affinities were unaltered. Thus, cGASP-1 behaves as a competitor for endogenous GASP-1 and the data are consistent with involvement of GASP-1 in the clozapine-mediated degradation of 5-HT$_{7(b)}$ receptors, likely through interaction that includes the C-terminus of the receptor.

Domains in the C-Terminus of the 5-HT$_7$ Receptor Regulate Clozapine- and Olanzapine-Mediated Downregulation. It was previously suggested by alanine substitutions of the β_1AR and the δ opioid receptor that GASP-1 interacts with certain residues within Helix-VIII (one hydrophobic and one basic residue within the start of Helix-VIII), common to a subset of GPCRs shown to interact with GASP-1.[17] At the 5-HT$_7$ receptor, these residues are LR (L391/R392) in the start of Helix-VIII. Additionally, Helix-VIII of the 5-HT$_7$ receptor is anchored by a palmitoylated C401,[40] which is flanked by two tyrosine-based YXXΦ motifs (Y is tyrosine and Φ is a residue with a bulky hydrophobic side chain; Figures 4A and 8). These YXXΦ motifs in the C-terminal tail of GPCRs have been suggested to be involved in ligand-stimulated internalization and lysosomal sorting,[4,41] and therefore, we hypothesize they may also recruit regulatory proteins, such as GASP-1, to mediate postendocytotic lysosomal sorting.

In order to determine the function of these different domains in the C-terminal tail of the 5-HT$_7$ receptor and test whether these are involved in recruitment of GASP-1 and lysosomal sorting of 5-HT$_{7(b)}$ receptors upon stimulation with clozapine or olanzapine, we generated alanine mutant receptors of L391/R392 and the two YXXΦ domains (395YRSL and 403YRNI) alone or in tandem. In addition, we tested the importance of palmitoylation-anchoring of Helix-VIII, by creating a serine mutant for the cysteine-anchor C401 (Figure 4B). Mutating either L391A/R392A, Y395A/L398A, Y403A/I406A, Y395A/L398A + Y403A/I406A, or C401S inhibited clozapine- and olanzapine-mediated degradation of 5-HT$_{7(b)}$ receptors (Figure 4C), demonstrating that specific amino acids within Helix-VIII and palmitoylation of Helix-VIII are critical for clozapine- and olanzapine-mediated downregulation. Surprisingly, a significant up-regulation of receptor density was observed in receptors

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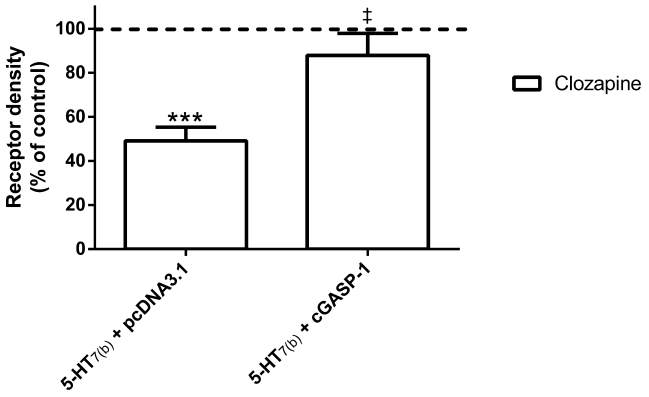

Figure 3. Overexpression of cGASP-1 inhibits clozapine-mediated degradation of 5-$HT_{7(b)}$ receptors. Receptor density (B_{max}) in membranes of HEK293 cells stably expressing the 5-$HT_{7(b)}$ receptor and transiently transfected with cGASP-1 or pcDNA3.1 preincubated 24 h with 1 μM clozapine, presented as percent of control (sister plates of cells preincubated without drug). B_{max} and K_d were determined by [^{3}H]5-CT binding, as described in Methods. The K_d of [^{3}H]5-CT for 5-$HT_{7(b)}$ receptors was not altered in either pcDNA3.1-transfected or cGASP-1 overexpressing cells, whether untreated or clozapine-treated. The B_{max} for untreated cells without (pcDNA3.1) and with transient overexpression of cGASP-1 were 10.8 ± 3.8 and 5.6 ± 1.6 pmol/mg protein, respectively. The data are mean ± SEM of 13 experiments. ***$p < 0.001$ compared to nontreated cells, $^{‡}p < 0.01$ compared to pcDNA3.1-transfected cells incubated with clozapine (Student's t test).

containing the Y395A/L398A mutation. The receptor mutations did not modify the binding affinities for [^{3}H]5-CT in the absence or presence of olanzapine or clozapine, but the mutated receptors displayed reduced expression (Table 1).

Signal Transduction Properties of 5-$HT_{7(b)}$ Mutant Receptors. We also tested to which extent the mutant receptors retained other functional properties by performing adenylyl cyclase (AC) assays to verify if the G protein activation was preserved. Based on the ratio between the ability to activate AC and receptor expression levels, the efficacies of the mutant receptors were 1.4 ± 0.3 for L391A/R392A, 0.4 ± 0.1 for Y395A/L398A, 0.6 ± 0.3 for C401S, 1.2 ± 0.3 for Y403A/I406A, and 0.2 ± 0.1 for Y395A/L398A + Y403A/I406A. The Y395A/L398A mutant and the double motif mutant (Y395A/L398A + Y403A/I406A) receptors showed a significantly reduced efficacy compared to WT receptors (Figure 5 and Table 2). Most interestingly, the Y403A/I406A and C401S mutants displayed about a 5-fold higher potency of 5-HT-stimulated AC activation compared to WT receptors (Figure 5 and Table 2), indicating altered G protein interaction in these mutant receptors.

The 5-$HT_{7(b)}$ receptors constitutively activate AC when stably or transiently expressed in HEK293 cells.[18] To determine whether the mutations modify the constitutive AC activity of 5-$HT_{7(b)}$ receptors, we compared the basal AC activity of the WT with mutant receptors and investigated the effect of the full inverse agonist (AC^{inv}) clozapine[18,19] to inhibit this basal AC activity. As shown in Figure 5 and in Table 2, Y403A/I406A, Y395A/L398A + Y403A/I406A, and C401S mutant receptors significantly increased basal AC activity relative to the WT receptor. However, the basal AC activity decreased by a similar percentage upon clozapine-stimulation in both WT and all mutated receptors (Table 2), including the Y395A/L398A + Y403A/I406A mutant, which only modestly displayed 5-HT-stimulated AC activity (Figure 5 and Table 2). In addition, we tested if clozapine were a full AC^{inv} by comparing its efficacy to reduce basal AC activity with that of olanzapine and methiothepin (an established full AC^{inv}[18]) in WT and all mutant receptors (Table 2). Our data indicate that clozapine is a full AC^{inv} in all receptors examined. In addition, 24 h preincubation with clozapine did not alter constitutive activity or inverse agonism in WT or mutated receptors (data not shown).

Structure of Mutated Residues in Helix-VIII (LR + YXXΦ Motifs). Next, we wanted to determine whether the mutated residues were constitutively accessible for interaction with GASP-1. Superimposing the inactive and active structures of the A_{2A} adenosine, β_2AR and M_2 muscarinic receptors showed that the residues corresponding to L391 (Leu8.50), R392 (Arg8.51) (both observed in 6.7% of class A GPCRs), Y395 (Tyr8.54) (observed only in ∼1% GPCRs), and L398 (Leu8.57) of the 5-HT_7 receptor are inaccessible from the intracellular side, but rather interact with and in-between TMI, II, and VII and face the lipid membrane and the indicated TM regions (Figure 6). This suggests that these amino acids are generally not constitutively accessible for GASP-1 interaction. Next, we wanted to determine if the alanine-substitutions altered the 3D conformation and/or the flexibility of Helix-VIII by ab initio modeling using the FloppyTail. Alanine-mutations did not unfold the helix, but changed the dynamic behavior of Helix-VIII (Figure 7). The most populated energetically feasible C-terminal tail conformations seem to be dominated by a similar α-helical formation in both WT and mutated receptors. However, the Y395A/L398A mutation, on average, yielded lowest energies, suggesting that it is the more flexible structure. Additionally, the most populated clusters of the Helix-VIII in the L391A/R392A mutant were pointing slightly more toward the cytosol, compared with the most populated clusters of the WT and Y395A/L398A mutation. The insertion of alanine did also not alter the relative position of that amino acid in the α-helical structure (Figure 7).

DISCUSSION

Here, we demonstrate that clozapine- and olanzapine-mediated degradation of 5-$HT_{7(b)}$ receptor is regulated by the C-terminal tail of the receptor. We show that 5-$HT_{7(b)}$ receptors interact with the sorting protein GASP-1 and that overexpression of cGASP-1, an inhibitor of endogenous GASP-1, prevent the clozapine- and olanzapine-mediated degradation. We also show that the clozapine- and olanzapine-mediated downregulation is regulated by domains in the C-terminal tail of the receptor since a C-terminal tail deletion in the receptor or mutations within or close to Helix-VIII of the receptor inhibited the downregulation. Additionally, the mutations in the C-terminal tail of the receptor modify the dynamic behavior and orientation of the 3D receptor structure, which may lead to biased signaling of clozapine and olanzapine.

Taken together, these data suggest that binding of the AC^{inv} clozapine and olanzapine to the 5-$HT_{7(b)}$ receptor leads to downregulation and lysosomal degradation by alteration of the equilibrium of receptor conformations that affect GASP-1-binding.

Clozapine- and Olanzapine-Mediated Downregulation Involves the C-Terminal Tail of 5-$HT_{7(b)}$ Receptors. The atypical antipsychotics clozapine and olanzapine are both D_2 and 5-HT_{2A} antagonists,[42] but also display high affinity for

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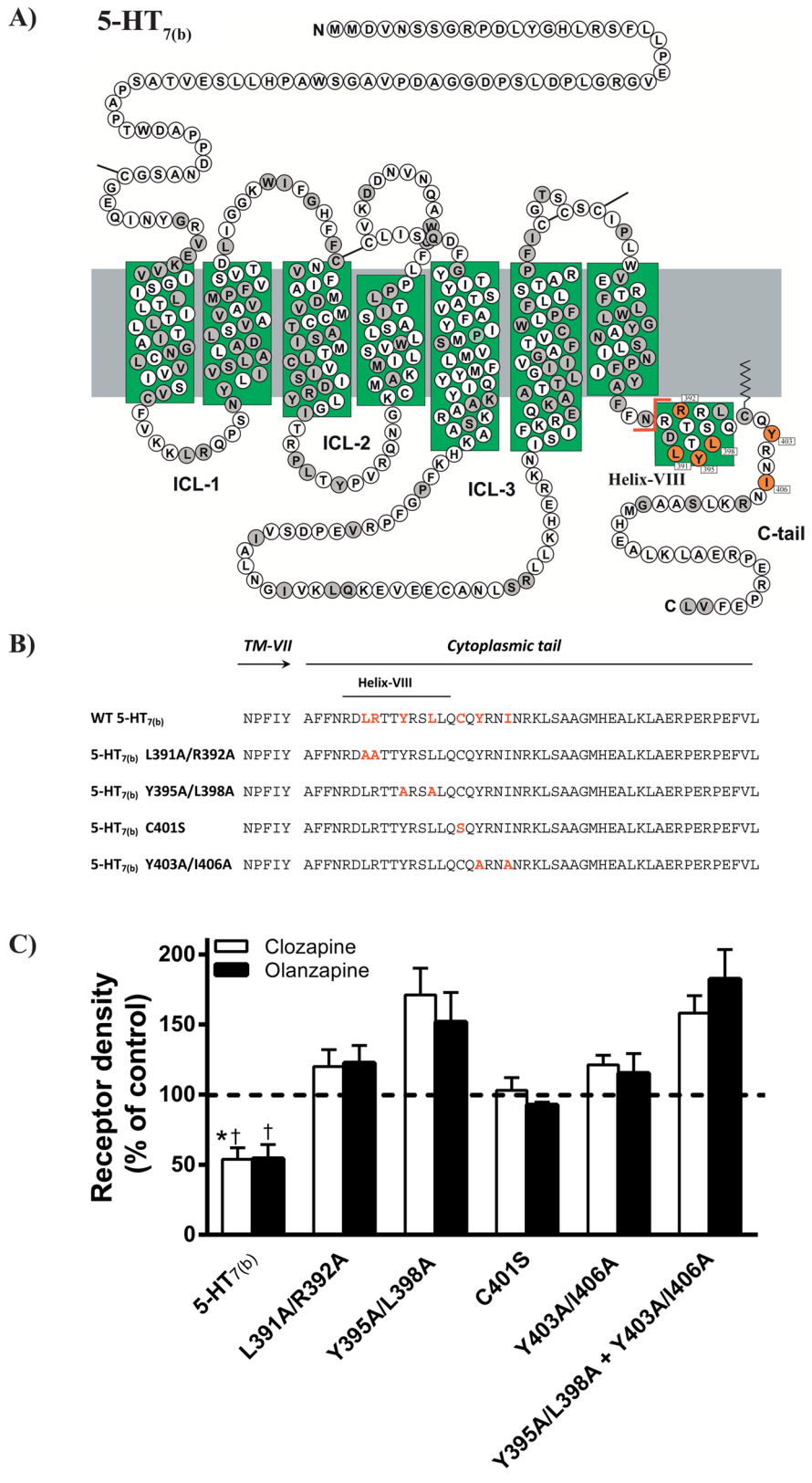

Figure 4. Sequence of the human 5-HT$_{7(b)}$ receptor. (A) Snake plot of the 5-HT$_{7(b)}$ receptor. Complete amino acid sequence of 5-HT$_{7(b)}$ receptor. Transmembrane helices TM-I to TM-VII and Helix-VIII in the C-terminal tail are indicated in green. Cysteine residues presumed to form disulfide bridges in the extracellular domain are indicated by black lines. Mutated amino acids, within or in proximity of Helix-VIII, for the L391A/R392A, Y395A/L398A, C401S, Y403A/I406A, and Y395A/L398A + Y403A/I406A 5-HT$_{7(b)}$ receptors are highlighted in orange. (B) C-Terminus of WT and mutated 5-HT$_{7(b)}$ receptors. Amino acid sequence starting from the NPFIY motif in TM-VII of WT 5-HT$_{7(b)}$ receptors. The mutated residues for L391A/R392A, Y395A/L398A, C401S, Y403A/I406A, and Y395A/L398A + Y403A/I406A 5-HT$_{7(b)}$ receptors are indicated in red with the corresponding substituted amino acid. (C) YXXΦ motifs, L391/R392 and C401 residues are involved in clozapine- and olanzapine-mediated degradation of 5-HT$_{7(b)}$ receptors. Receptor density (B_{max}) in membranes of QBI HEK293 cells expressing the WT 5-HT$_{7(b)}$, 5-HT$_{7(b)}$ L391A/

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Figure 4. continued

R392A, 5-HT$_{7(b)}$ Y395A/L398A, 5-HT$_{7(b)}$ C401S, 5-HT$_{7(b)}$ Y403A/I406A, and 5-HT$_{7(b)}$ Y395A/L398A + Y403A/I406A receptors preincubated 24 h with 1 μM clozapine or 20 μM olanzapine, presented as percent of control (sister plates of cells preincubated without drug). B_{max} was determined by [^{3}H]5-CT binding as described in Methods. Data are the mean ± SEM of 4−10 experiments. *$p < 0.05$ compared to nontreated cells, $^{\dagger}p < 0.05$ versus all 5-HT$_{7(b)}$ mutant receptors incubated with clozapine or olanzapine (two-way ANOVA with Bonferroni's multiple comparison test).

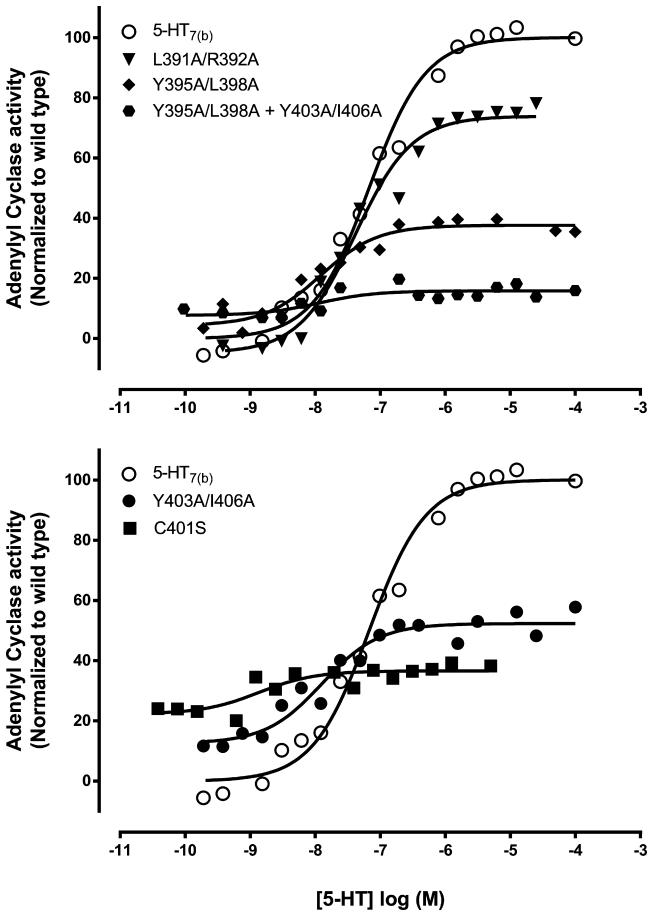

Figure 5. 5-HT-mediated activation of AC by the FLAG-tagged WT, L391A/R392A, Y395A/L398A, C401S, Y403A/I406A, and Y395A/L398A + Y403A/I406A 5-HT$_{7(b)}$ receptors. Membranes from QBI-HEK293 cells transiently expressing the indicated FLAG 5-HT$_{7(b)}$ receptor variants were incubated in the presence of increasing concentration of the agonist 5-HT. Concentration−response curves are shown as percent of maximal 5-HT-stimulated activity in the WT 5-HT$_{7(b)}$ receptors. AC activity was measured as described in Methods, and data shown are representative of 7−14 independent experiments. Mean values for EC$_{50}$ are shown in Table 2.

many other receptors, including 5-HT$_7$ receptors,[21,23] suggested to be involved in the treatments effect in schizophrenia.[43]

We previously demonstrated that clozapine and olanzapine are biased ligands toward the 5-HT$_{7(b)}$ receptor where they behave as AC^{inv}, but induce internalization followed by lysosomal degradation of the receptors upon prolonged exposure.[23] Similarly, clozapine has also been shown to induce a slow downregulation of 5-HT$_{2A}$ receptors, despite showing inverse agonism on G protein activation.[44] Possibly, this property may be important for the slow onset of antipsychotic effect or the effect itself of clozapine.

In initial experiments aimed at determining which part of the 5-HT$_{7(b)}$ receptor were involved in the clozapine- and olanzapine-mediated downregulation, we found that receptors lacking the C-terminal tail did not show downregulation after 24 h of incubation with clozapine or olanzapine (Figure 1). This suggested a crucial importance of the C-terminal tail in the downregulation of 5-HT$_{7(b)}$ receptors. Moreover, we have previously demonstrated that 5-HT$_{7(b)}$ receptors C-terminally tagged with YFP are resistant to clozapine-mediated lysosomal degradation.[23] Together, these findings suggested that 5-HT$_{7(b)}$ receptor degradation is dependent on docking of protein(s) to specific domains in the C-terminal tail (masked by the presence of C-terminal YFP), targeting the receptor to a degradative pathway after binding clozapine or olanzapine. This is similar to the δ opioid receptor, where the C-terminal tail determines the degradative pathway.[9]

We further investigated which regulatory domains present in the C-terminal tail of the 5-HT$_{7(b)}$ receptor could be involved in the clozapine- and olanzapine-mediated receptor downregulation by testing the effect of different mutations in the 5-HT$_{7(b)}$ receptor C-terminal tail.

We selected two YXXΦ motifs, two residues suggested to be common among GPCRs interacting with GASP-1[17] and a palmitoylated cysteine anchor, all present in or close to the Helix-VIII, which has been shown to be important for interaction with GASP-1.[7] We constructed and expressed 5-HT$_{7(b)}$ receptors with the mutations L391A/R392A, Y395A/L398A, Y403A/I406A, and C401S in the C-terminal tail of the human 5-HT$_{7(b)}$ receptor. All these mutations inhibited clozapine- and olanzapine-mediated downregulation (Figure 4 C), suggesting that the residues chosen in and around Helix-VIII are important in regulating ligand-mediated receptor downregulation. More distal mutations, such as deletion of the extreme PDZ domain of the 5-HT$_{7(b)}$ receptor (Δ430FVL) did not change the clozapine-mediated downregulation, demonstrating that not all mutations in the C-terminal tail of 5-HT$_{7(b)}$ receptor interfere with receptor trafficking.[23] The loss of ligand-stimulated downregulation in the 391L$_{8.50}$A/392R$_{8.51}$A mutant is similar to that demonstrated for the 8.50/8.51 mutation in the β_1AR and δ opioid receptors, where these were suggested to be residues important for GASP-1 binding.[17] As illustrated in Figure 6, Phe/Leu in 8.50 and Arg/Lys in 8.51 is present in a majority of GPCRs (59% have both), including the β_1AR, 5-HT$_7$ and δ opioid receptors, that interact with GASP-1, and the μ opioid receptor which does not interact with GASP-1,[9] suggesting that these residues are not sufficient for binding to GASP-1.

5-HT$_{7(b)}$ Receptor Trafficking upon Clozapine-Incubation Is Mediated by GASP-1. A prime candidate for a sorting protein interacting with the C-terminal tail of the 5-HT$_{7(b)}$ receptor and possibly involved in the ligand-mediated degradation could be a protein of the G protein-associated sorting protein (GASP) family.[17] The members of the GASP family are shown to associate with a broad range of GPCRs and are involved in the postendocytotic sorting of multiple receptors.[8,13,17] One member of this family, GASP-1, has been shown to be involved in downregulation of different GPCRs,[9−12,14,15,45] and shown to bind a GST-tagged C-terminal tail of the 5-HT$_{7(a)}$ receptor.[17,39] Using coimmuno-

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Table 2. Mutations in the C-Terminal Tail of the 5-HT$_{7(b)}$ Receptor Increase Basal Adenylyl Cyclase Activity and Modify Potency of 5-HT[a]

FLAG-tagged 5-HT$_{7(b)}$ receptor	5-HT pEC_{50}	5-HT efficacy (% of WT)	receptor density (% of WT)	basal AC activity (% above WT)	constitutive activity (% of basal)	olanzapine efficacy (% of clozapine)	methiothepin efficacy (% of clozapine)
WT	7.1 ± 0,1	100			53 ± 6	97 ± 10	101 ± 2
L391A/R392A	7.5 ± 0.1	56 ± 9	41 ± 7	−4 ± 6	43 ± 5	100 ± 21	106 ± 12
Y395A/L398A	7.7 ± 0.2	28 ± 3[d]	65 ± 12	25 ± 10	61 ± 5	104 ± 30	101 ± 2
C401S	8.3 ± 0.4[c]	27 ± 7	42 ± 13	83 ± 14[b]	56 ± 6	88 ± 18	96 ± 27
Y403A/I406A	7.9 ± 0.1[b]	75 ± 17	61 ± 5	44 ± 11[b]	58 ± 8	96 ± 2	90 ± 1
Y395A/L398A + Y403A/I406A	7.4 ± 0.4	8 ± 4[d]	50 ± 6	41 ± 8[c]	58 ± 8	83 ± 4	94 ± 22

[a]Adenylyl cyclase activity in membranes from transiently transfected QBI-HEK293 cells was measured in the absence (basal AC activity) and in the presence of increasing concentration of agonist (5-HT). Potency (pEC_{50}) was then calculated as described in Methods. Receptor density and efficacy (maximal AC activity above basal) was calculated as percent of WT 5-HT$_{7(b)}$ receptors. The basal AC activity for the mutant receptors is presented as percent above WT 5-HT$_{7(b)}$ receptors. The basal AC activities of the different FLAG-tagged 5-HT$_{7(b)}$ receptor variants were (in pmol/mg protein/min): WT, 15.1 ± 1.9; L391A/R392A, 11.9 ± 2.0; Y395A/L398A, 17.9 ± 2.2; C401S, 20.4 ± 1.8; Y403A/I406A, 24.4 ± 3.9; and Y395A/L398A + Y403A/I406A, 21.3 ± 3.0. The constitutive activity was defined as that part of the basal AC activity inhibited by 10 μM clozapine and reported as percent of the basal AC activity. The efficacy of 20 μM olanzapine or 1 μM methiothepin (a full inverse agonist[18]) to reduce basal AC activity was compared to that of 10 μM clozapine. Data shown are mean ± SEM from 2 to 12 experiments. [b]$p < 0.05$. [c]$p < 0.01$ versus WT (one-way ANOVA with Bonferroni's multiple comparison test). [d]$p < 0.05$ versus their respectively receptor density (two-way ANOVA with Bonferroni's multiple comparison test).

precipitation, we verified an interaction between the full-length 5-HT$_{7(b)}$ receptor and the C-terminus of and full length GASP-1 protein (Figure 2). To demonstrate the involvement of GASP-1 in the clozapine-mediated downregulation of 5-HT$_{7(b)}$ receptors, we overexpressed cGASP-1 as a competitor for endogenous GASP-1[9] in cells stably expressing 5-HT$_{7(b)}$ receptors and incubated with clozapine for 24 h and found that the clozapine-mediated downregulation of 5-HT$_{7(b)}$ receptors was prevented (Figure 3). A stronger argument for the involvement of GASP-1 could be obtained using siRNA- or shRNA-mediated knock down of GASP-1, but despite several previous reports successfully knocking down GASP-1 by siRNA or shRNA,[14−16] in our hands GASP-1 was not sufficiently knocked down by several shRNA or siRNA constructs in HEK293 cells to obtain conclusive results. Other members of the GASP family (GASP-2, -3, and -7) have also been shown to bind the C-terminal tail of the 5-HT$_{7(a)}$ receptor, and could also traffic different receptors to lysosomes.[39] Taking into consideration that cGASP-1 has high sequence similarities with at least GASP-2 (77% identical amino acids shared with cGASP-1),[17,39] the trafficking of the 5-HT$_{7(b)}$ receptor to the lysosomes could be mediated, not only by GASP-1, but also by GASP-2.

Role of Domains in the C-Terminus of 5-HT$_{7(b)}$ Receptor Involved in Clozapine- And Olanzapine-Mediated Downregulation. As previously discussed, mutating residues 8.50 and 8.51 has been shown to decrease GASP-1-binding in the β_1AR and δ opioid receptor.[17] Similarly, mutating these amino acids in the 5-HT$_{7(b)}$ receptor prevented clozapine-mediated downregulation (Figure 4C). Structure data indicate that these conserved residues are facing the lipid bilayer and rather interacting with TMI, II and VII (Figure 6). The ab initio modeling suggests that the structures of Helix-VIII in the WT 5-HT$_{7(b)}$ receptor and the receptors mutated at L391A/R392A (8.50*A*/8.51A) and at Y395A/L398A (8.54*A*/8.57A) are rather similar (Figure 7). Helix-VIII is amphipathic, with a hydrophobic side facing the membrane bilayer and a polar side facing the cytosol. Both L391 and Y395/L398 face toward the membrane lipid bilayer and alanine-mutations did not disrupt the relative placement of these residues, nor the α-helical structure (Figure 7). This appears reasonable, as alanine mutations are known to stabilize helix formation. In summary, full flexible modeling of Helix-VIII suggests that the exposure state of the residue positions 8.50A, 8.51A, 8.54A, and 8.57A is unchanged similar to the various more than 25 crystal structures which also have different hydrophobic/aromatic residues in these positions and still have highly similar superimposable Helix-VIII structures. Our modeling also suggests that there are differences in the dynamic behavior between WT, L391A/R392A, and Y395A/L398A. Whereas L391A/R392A and WT have a similar RMSD versus energy profile, the minimum of Y395A/L398A is clearly shifted compared to WT by 1 Å RMSD. More interestingly, the energy of the best-scored models reveals that Y395A/L398A on average has lower energies compared to WT and L391A/R392A, suggesting that L391A/R392A is more flexible (Figure 7). That the L391A/R392A mutation displays similar phenotypes to the L391A/R392A, yet displays differences in ab initio modeling implies that it is not clear how these residues are involved in clozapine- and olanzapine-mediated degradation of 5-HT$_{7(b)}$ receptors. What is clear, is that all these residues (in the WT) point toward TMI and TMVII and possibly interact with residues there that could be involved in stabilizing different receptor conformations, but this has not been examined in the current modeling data. From this we can conclude that either clozapine-binding to the 5-HT$_7$ receptor exposes these residues for GASP-recruitment or that mutations in L391, R392, Y395, and L398 will change the dynamic behavior of different TMI and TMVII packing and orientation thus causing indirect effects on receptor function, potentially disrupting clozapine-stabilized receptor conformations that recruit GASP-1. Minor mutations at the 5-HT$_{2B}$ receptor were also shown to induce biased signaling and/or stabilize active/inactive receptor states.[46] The effects of L391A/R392A and Y395A/L398A could therefore be indirect and this implies that GASP-1 does not necessarily interact with these particular residues, but mutations in these residues constitutively expose other residues important for GASP-1-binding, leading to no additional regulation by adding clozapine or olanzapine. In accordance with this, we have found that all mutant receptors bound cGASP-1 in an in vitro coimmunoprecipitation experiment (data not shown). This would explain the significantly

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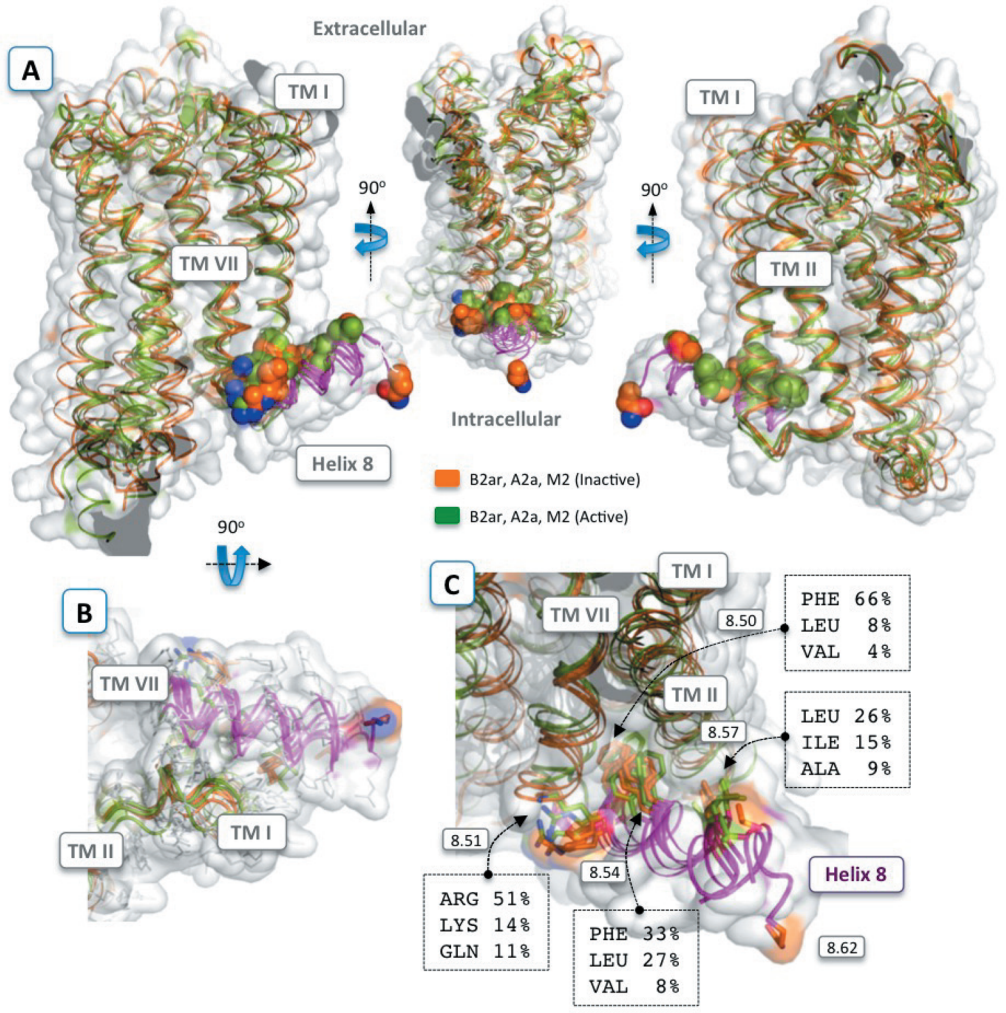

Figure 6. Structures of Helix-VIII. (A) Side view of the superimposed inactive (orange) and active (green) structures of the β_2AR, A_{2A} adenosine and M_2 muscarinic receptors (PDB ID: 3EML, 2RH1, 3UON, 3QAK, 3SN6, and 4MQS). The molecular exteriors are shown by white transparent surfaces. Helix-VIII (purple cartoon) and the conserved residue positions 8.50, 8.51, 8.54, and 8.57 (orange and green spheres), corresponding to mutated residues Leu391, Arg392, Tyr395, and Leu398 in the $5HT_7$ receptor, adopt a highly similar conformation in the active and inactive β_2AR, A_{2A}, and M_2 structures. Residue position 8.62 (corresponding to Tyr403 in 5-HT_7) is only resolved in the inactive A_{2A} structure (PDB ID: 3EML) as the C-terminal residue. Its structural location is most likely uncertain. The residue position 8.65 (corresponding to Ile403 in $5HT_7$) is not resolved in any of the crystal structures. (B) Intracellular view of TMI, -II, -VII and Helix-VIII showing that the mutated positions 8.50, 8.51, 8.54, and 8.57 are inaccessible from the intracellular side. (C) Extracellular close-up view of Helix-VIII showing that the residue positions 8.50, 8.51, 8.54, and 8.57 interact with and in between TMI, -II, and VII and face the lipid membrane. Numbers indicate the frequency of the three most observed amino acids in those positions in Helix-VIII among 283 human nonolfactory rhodopsin-like receptors.

lower receptor density observed in all mutant receptors due to constitutive GASP-1 recruitment and sorting to lysosomes of constitutively internalized receptors that is not enhanced by adding clozapine or olanzapine. However, it has been reported that cGASP-1 interacts better with the receptor C-terminal tail than the full-length GASP-1,[7] suggesting that cGASP-1 could coimmunoprecipitate differently from GASP-1. The previously mentioned alanine-mutation of F8.50 and R/K8.51 in β_1AR and δ opioid receptor only *reduced* GASP-1-binding, suggesting that there could be a destabilization of Helix-VIII resulting in reduced GASP-1 binding.[17] Likewise, the C401S mutation would prevent palmitoylation and correct membrane embedment of Helix-VIII, also constitutively exposing GASP-1-binding.

How the Y403A/I406A mutation also prevented clozapine- and olanzapine-mediated downregulation is less clear, as there is no crystal structure information available to give any clues as to the position of the 403YRNI domain. Speculatively, mutations here might prevent palmitoylation of C401, thus preventing proper orientation of Helix-VIII, similar to the C401S mutation. It is more than conceivable that the C401S and Y403A/I406A mutations adopt similar conformations, as similar results were obtained in AC assays only for these mutations. Mutations in both 395YRSL and 403YRNI motifs affected downregulation comparably to mutation in a single YXXΦ motif, indicating that mutations can be related to the same effect. Together, this suggests that Helix-VIII is critically involved in the regulation of receptor trafficking, and L391/R392, YXXΦ, and C401 mutations may alter the tertiary structure of Helix-VIII, allowing constitutive exposure to GASP-1, and thereby inhibit the clozapine- and olanzapine-mediated downregulation of 5-$HT_{7(b)}$ receptors as receptors are constitutively downregulated.

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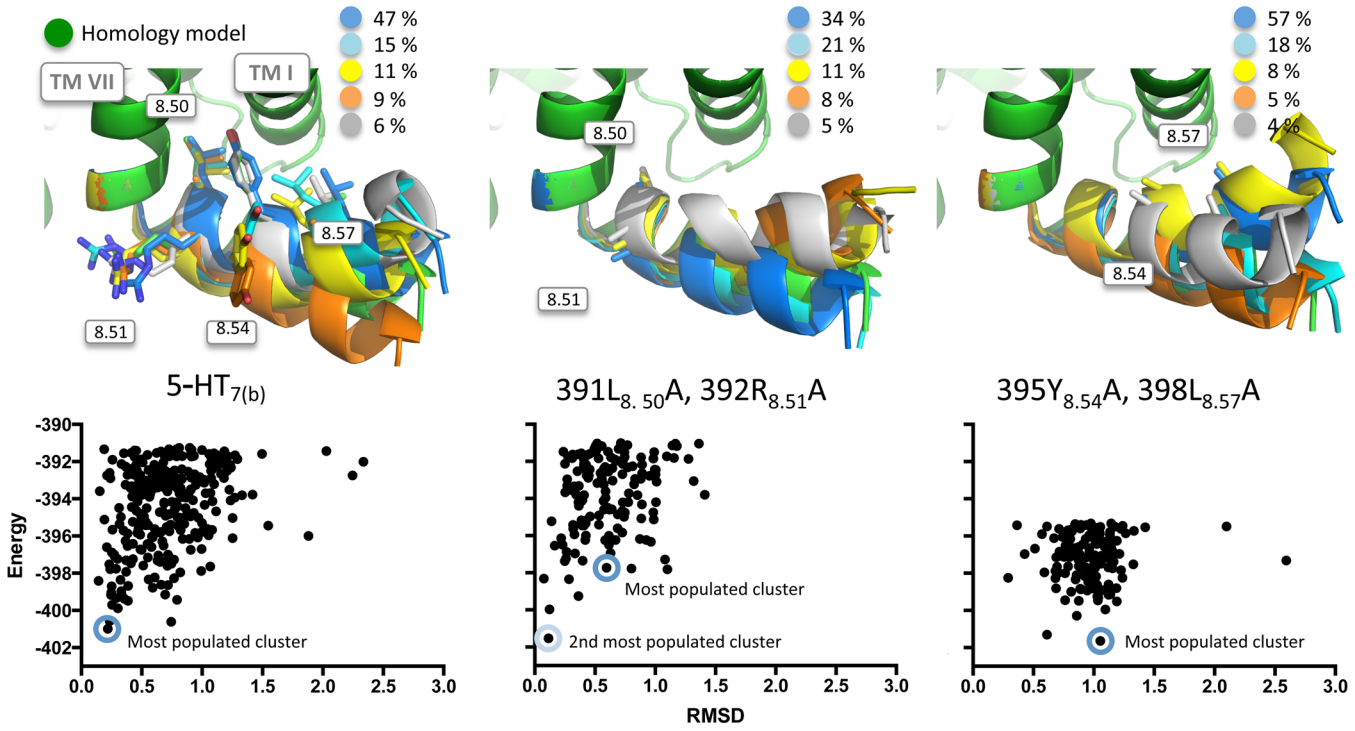

Figure 7. Ab initio modeling of the Helix-VIII of the 5-HT$_{7(b)}$ receptor. The Rosetta FloppyTail protocol was used for ab initio modeling of the C-terminal (Helix-VIII) tail (N388 to Y403) of the 5-HT$_{7(b)}$ receptor and the two mutated receptor variants (391L$_{8.50}$A and 392R$_{8.51}$A) and (395Y$_{8.54}$A and 398L$_{8.57}$A) using the procedure described in Methods. The protocol was used for testing hypotheses about possible conformations and exploring accessible conformation space. The Helix-VIII tail is assumed to be flexible, with no "one true conformation". The upper panel shows the nonredundant representative models of the five most populated (blue, light blue, yellow, orange, and gray) Helix-VIII conformations of the WT and the two mutated receptor variants. By comparison, the best-scored refined homology model of the 5-HT$_{7(b)}$ receptor is shown in green. The RMSD of backbone atoms for the Helix-VIII of the top 5% best-scored models (generated by the FloppyTail protocol) compared to the best-scored homology model as a function of the Rosetta Energy [Rosetta energy units] is shown in the lower panel. The most populated energetically feasible C-terminal tail conformation is dominated by α-helix formation in the WT receptor as well as in the mutated receptor variants.

Functional Role of C-Terminal Domains of the 5-HT$_{7(b)}$ Receptor. While we found Helix-VIII of the 5-HT$_{7(b)}$ receptor to be a critical structural determinant for the ligand-induced interaction with GASP-1, Helix-VIII is also implicated in the interaction with G proteins.[47−50] Therefore, we tested the ability of the 5-HT$_{7(b)}$ mutant receptors to bind ligands and activate adenylyl cyclase. There was unaltered affinity for ligand in all mutant receptors and all receptors displayed similar constitutive activity. The efficacy of clozapine as an AC^{inv} was found to be similar to the efficacy of the full AC^{inv} olanzapine and methiothepin[18,23] in all mutated receptors (Table 2). However, the Y403A/I406A, Y395A/L398A + Y403A/I406A, and C401S 5-HT$_{7(b)}$ receptors displayed increased basal AC activity compared to WT receptors, but this increased basal AC activity was not reflected in a larger fraction representing constitutive activity (Table 2), which is in agreement with previous reports examining this on 5-HT$_7$ receptors.[18,19,40,51] Mutations may change the conformation of the C-terminal tail which could modify the constitutive and 5-HT-stimulated AC activity of the 5-HT$_{7(b)}$ receptor, due to a different capacity of the C-terminal tail to bind and activate G protein. The cysteine can undergo post-translational modification with long-chain saturated fatty acids (palmitate), which forms the forth intracellular loop of the receptor. Palmitoylation has been shown to regulate basal AC activity of the mouse 5-HT$_{7(a)}$ receptor, where mutation of the C-terminal cysteine residue 404(8.60) (corresponding to the C401(8.60) at the human 5-HT$_{7(b)}$) increased basal AC activity and increased the potency of 5-HT.[40] We found similar observations for the C401S mutant, indicating that human and mouse 5-HT$_7$ receptors are similarly palmitoylated which has implications for G protein activation (Figure 5). In our study, the Y403A/I406A mutant receptors behaved as the C401S mutant receptor, inducing an increase of basal AC activity and increased the potency of 5-HT (Figure 5 and Table 2). Therefore, we suggest that the 403YRNI-mutated motif could hinder the cysteine from being palmitoylated, and thereby increase the basal AC activity and increase the potency, as observed in the mouse 5-HT$_{7(a)}$ mutant receptors lacking palmitoylation sites.[40]

In addition, mutating the 395YRSL motifs reduced the efficacy to activate AC, indicating that this alters G protein activation. Similarly, the double motif mutation displayed an increased basal AC activation (probably due to the 403YRNI mutation) and a very low efficacy (probably due to the 395YRSL mutation), despite displaying normal ligand-binding. This could be due to 395YRSL stabilizing receptor conformations that interact with G protein, since this motif when mutated appears to form the most flexible structure (Figure 7). Lack of palmitoylation could modify the capacity to form Helix-VIII, which may play an important role in the acquisition of the receptor conformations involved in G protein activation.[49] Therefore, mutations in both YXXΦ motifs change the C-terminal tail conformation, possibly through alterations of palmitoylation, modifying the capacity to form Helix-VIII and interfere with the interaction between the C-terminal tail and G protein. In this respect, both GASP-1 interaction and proper G

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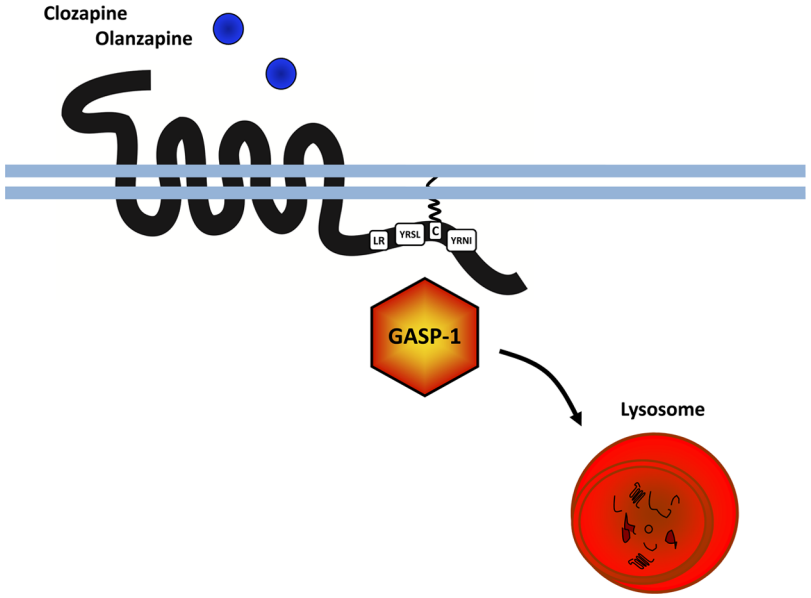

Figure 8. Hypothetical model of 5-HT$_{7(b)}$ receptor trafficking upon clozapine- and olanzapine-stimulation. Clozapine and olanzapine, biased ligands toward the 5-HT$_{7(b)}$ receptor, change the conformation of the C-terminal tail and facilitate the recruitment of the sorting protein GASP-1 and subsequent receptor downregulation.

protein activation require a correctly aligned and palmitoylated Helix-VIII.

CONCLUSION

We conclude that the considered residues in the C-terminal tail are essential for clozapine- and olanzapine-mediated down-regulation and degradation, which appears to be lysosomal in a GASP-dependent manner. In addition, a rearrangement of the C-terminal tail by mutations at these residues seems to modify interaction with G protein. Clozapine and olanzapine behave as functionally selective ligands and their effect is altered by the mutations at the C-terminal tail (Figure 8).

METHODS

Materials. All ligands and chemicals were from Sigma-Aldrich (St. Louis MO), unless otherwise noted. Olanzapine (Zyprexa ad injectibilia) was from Eli Lilly and Co. (Indianapolis, IN). Methiothepin maleate was from Tocris Bioscence (Bristol, U.K.). G418, penicillin-streptomycin, L-glutamine, LipofectAMINE LTX, and LipofectAMINE 2000 were from Invitrogen Life Technologies (Carlsbad, CA). The radiochemicals 5-carboxamido[^{3}H]tryptamine trifluoroacetate ([^{3}H]5-CT) (14−106 Ci/mmol) and [α-^{32}P] ATP (400 Ci/mmol) were from PerkinElmer (Waltham, MA). The antibodies mouse anti-FLAG M2 and rabbit anti-GFP were from Sigma-Aldrich; the antibodies goat anti-rabbit IRDye680 and goat anti-mouse IRDye680 were from LI-COR Biosciences.

Plasmids. Plasmids encoding the human (h) FLAG-tagged 5-HT$_{7(b)}$ receptor and 5-HT$_7$ ΔC-tail (truncated after F386) have previously been described.[52] The FLAG-5-HT$_{7(b)}$ wild type (WT) served as a template to generate the following mutant receptors by an overlapping extension PCR technique: the mutant 5-HT$_{7(b)}$ C401S, with the substitution of Ser for Cys401 (nucleotide G1202C) and 5-HT$_{7(b)}$ LR391AA with the substitution of Ala for Leu and Arg (nucleotides CT1171−1172GC and AG1174−1175GC). The 5-HT$_{7(b)}$ receptors with mutations at YXXΦ motifs were constructed introducing mutations individually or in combination with the substitution of Ala for Tyr395, Leu398, Tyr403, and Ile406. We used the following substitution of nucleotides: TA1183−1184GC and CT1192−1193GC for Y395A/L398A and TA1207−1208GC and AT1216−1217GC for Y403A/I406A. All mutant 5-HT$_{7(b)}$ receptors were in pcDNA3.1 and verified by DNA sequencing. Plasmids encoding HA-cGASP-1, GASP-1-GFP, and cGASP-1-GFP in pcDNA3.1 were kindly provided by Dr. Jennifer Whistler (Ernest Gallo Clinic and Research Center, University of California, San Francisco, CA) and have been described previously.[9] The cGASP-1 is corresponding to the C-terminal 497 residues of GASP-1 and is a competitor for endogenous GASP-1 at GPCRs.

Transfection of HEK293 Cells. HEK293, QBI-HEK293A, and HEK293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were transiently transfected using LipofectAMINE 2000 and Lipofectamine LFX according to the manufacturer's protocol or with polyethyleneiminie (PEI) as previously described,[53] with the indicated plasmids. After transfection, cells were cultured in 5-HT-free medium, UltraCULTURE (Lonza, Basel, Switzerland), supplemented with L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) for 48 h. HEK293 cells stably expressing h5-HT$_{7(b)}$ receptors[21] were continuously grown in UltraCULTURE and transiently transfected using LipofectAMINE 2000 according to the manufacturer's protocol.

Membrane Preparation, Radioligand Binding, and Adenylyl Cyclase Assay. Membranes were prepared as described previously.[21] For experiments with preincubation with clozapine and olanzapine, the cells were subjected to a more vigorous washing protocol (three washes within 2 h) to remove residual drug, as previously described.[37] Radioligand binding and adenylyl cyclase assays on membrane preparations were performed as previously described,[21] estimating K_d and B_{max} based on saturation binding experiments with increasing concentrations of [^{3}H]5-CT, as previously described.[21]

Coimmunoprecipitation. HEK293T cells were transfected, washed after 48 h with PBS, collected, and frozen at −70 °C. Next, cells were lysed in 400 μL of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 0.1% SDS, and 0.5% deoxycholic acid) supplemented with protease and phosphatase inhibitors (2.5 μg/mL aprotinin, 1 mM PEFA-block, 10 μg/mL leupeptin, and 10 mM β-glycerolphosphate) for 1 h rotating at 4 °C. A 40 μL lysate sample was denatured at 37 °C for 10 min in SDS-sample buffer (4% SDS, 50% glycerol, 0.2% bromophenol blue, 65 mM Tris-HCl pH 6.8, and 50 mM dithiothreitol) and loaded on a 10% SDS-PAGE gel to check the protein expression. To the rest of the lysate, 2 μg of primary antibody (mouse anti-FLAG M2) was added. After 4 h rotation at 4 °C, 20 μL of protein A trisacryl beads (Thermo Scientific) were added and

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further rotated overnight at 4 °C. Beads were then washed three times with RIPA buffer and subsequently denatured at 37 °C for 10 min in SDS-sample buffer. Proteins were separated on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Immunodetection was performed with the indicated primary antibodies diluted 1:2000 and secondary antibodies diluted 1:15000 and developed with the Odyssey Infrared Imaging system (LI-COR Biosciences).

Modeling of the C-Terminal Tail (Helix-VIII). Ab initio structure prediction methods were used to generate three-dimensional models of the C-terminal tail for the WT 5-$HT_{7(b)}$ receptor and two double mutated receptor variants ($391L_{8.50}$A and $392R_{8.51}$A) and ($395Y_{8.54}$A and $398L_{8.57}$A). The sequence of the human 5-$HT_{7(b)}$ receptor was obtained from www.uniprot.org (accession code P34969). First, we constructed a homology model of the human 5-$HT_{7(b)}$ receptor, based on the high-resolution crystal structures of 5-HT_{1B} (PDBcode 4IB4)[54] and 5-HT_{2B} receptors (PDB code 4IAR)[46] as structural templates using the ICM package (version 3.7-2, Molsoft LLC, San Diego, CA). Second, we produced 100 energetically feasible receptor conformations by minimizing the energy of the backbone and repacking side chains using the Rosetta relax protocol (version 3.4) and the membrane force field.[55,56] The best-scored model was then applied in a two-stage modeling protocol. In the first step, two double mutated receptor variants ($391L_{8.50}$A and $392R_{8.51}$A) and ($395Y_{8.54}$A and $398L_{8.57}$A) were constructed. In the second step, the Rosetta FloppyTail protocol was used to generate 2000 structural models of the C-terminal tail (residue 388N to 403Y) of the WT receptor and the mutated receptor variants, added in an extended conformation (±135° phi, psi angles), using the parameters -ex1, -ex2, use_input_sc, frequency of perturb_cycles=2000, and refine_cycles=1000. The remaining C-terminal residues (404R−432L) were not modeled. Finally, the top 5% best scoring FloppyTail models (for WT, and the double mutated receptor variants), were clustered based on root-mean-square deviation (RMSD) of backbone atoms for the C-terminal residues (388N to 403Y) using the Rosetta clustering routine and a 1 Å threshold.

Protein Measurements. The protein concentration in the membrane preparations was measured with the Micro BCA Assay Reagent Kit (Uptima) using bovine serum albumin as a standard.

Statistics. Statistical significance ($p < 0.05$, $p < 0.01$, or $p < 0.001$) was determined using GraphPad Prism 6.0 for Windows (GraphPad Software, San Diego, CA) with the statistical methods indicated.

Nomenclature. We adopted the nomenclature proposed by ref 57 to provide clarity and to differentiate between agonism and inverse agonism upon the measured responses in the current work. AC^{inv} is therefore inverse agonism on AC activation.

The 5-$HT_{7(b)}$ receptors mutated at L391, R392, Y395A, L398A, and C401S were indicated either with the amino acids numbering or with Ballesteros Weinstein numbering ($391L_{8.50}$, $392R_{8.51}$, $395Y_{8.54}$, $398L_{8.57}$, and $401C_{8.6}$). The 5-$HT_{7(b)}$ receptors mutated at Y403A, I406A were outside the Helix-VIII and, therefore, are denoted only by their amino acid number.

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Participated in research design: Manfra, Van Craenenbroeck, Frimurer, Schwartz, Levy, and Andressen. Conducted experiments: Manfra, Van Craenenbroeck, Skieterska, Frimurer, and Andressen. Performed analysis: Manfra, Van Craenenbroeck, Skieterska, Frimurer, Schwartz, and Andressen. Wrote or contributed to the writing of the manuscript: Manfra, Van Craenenbroeck, Skieterska, Frimurer, Schwartz, Levy, and Andressen.

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Notes

The authors declare no competing financial interest.

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