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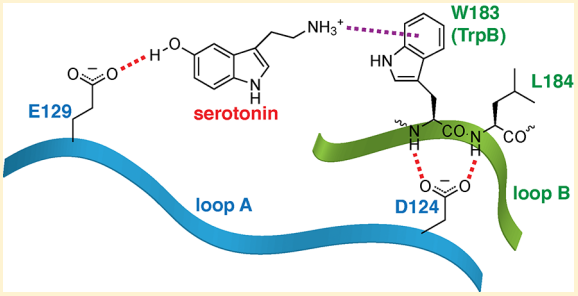
A Coupled Array of Noncovalent Interactions Impacts the Function of the 5-HT$_3$A Serotonin Receptor in an Agonist-Specific Way

Timothy F. Miles,[†] Kiowa S. Bower,[‡] Henry A. Lester,[†] and Dennis A. Dougherty*[,‡]

[†]Division of Biology and [‡]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, United States

ABSTRACT: The serotonin type 3A (5-HT$_3$A) receptor is a Cys-loop (pentameric) neurotransmitter-gated ion channel found in the central and peripheral nervous systems and implicated in numerous diseases. In previous studies with the endogenous agonist serotonin, we identified two interactions critical for receptor function: a cation−π interaction with W183 in loop B (TrpB) and a hydrogen bond to E129 in loop A. Here we employ mutant cycle analyses utilizing conventional and unnatural amino acid mutagenesis to demonstrate that a third residue, D124 of loop A, forms two functionally important hydrogen bonds to the backbone of loop B. We also show that these three interactions, the cation−π interaction, the backbone hydrogen bonds, and the E129 hydrogen bond, are tightly coupled to each other, suggesting they function as a single unit. We also identify key functional differences between serotonin and the competitive partial agonist *m*-chlorophenyl biguanide (mCPBG) at these residues. mCPBG displays no cation−π at TrpB and extreme sensitivity to the positioning of E129, on which it is reliant for initiation of channel gating.



KEYWORDS: *Serotonin, mCPBG, Cys-loop, binding site, gating, unnatural amino acid mutagenesis*

INTRODUCTION

The serotonin type 3 receptor (5-HT$_3$) is a Cys-loop (pentameric) neurotransmitter-gated ion channel whose activation elicits fast excitatory responses.[1,2] The 5-HT$_3$ receptor is expressed in both the central and peripheral nervous systems and is known to be involved in multiple psychiatric and neurological disorders.[3] Five distinct subunits of the 5-HT$_3$ receptor have been identified,[4] but only the A subunit forms functional homomeric receptors. These subunits, consisting of a large N-terminal extracellular domain, four transmembrane helices (M1−4), and a large intracellular loop (M3−M4), arrange into a symmetrical pentamer around a central pore.

As with other pentameric receptors, the ligand binding domain is located at the interface between the extracellular domains of two adjacent subunits. More specifically, the binding site is composed of six loops from both the principle (A−C) and complementary (D−F) faces. Agonist binding leads to an opening of the channel gate at the M2 helix, over 60 Å distant.

In previous studies of the 5-HT$_3$A receptor, we identified critical roles for two residues. W183 on loop B (termed TrpB) makes a cation−π interaction to serotonin,[5] playing the same role as the aligning residue in most nicotinic acetylcholine receptors (nAChR).[6−8] E129 on loop A of the 5-HT$_3$ receptor makes a hydrogen bond that is critical to receptor function.[9] We proposed that the hydrogen bond occurs with the OH of the agonist, serotonin. The aligning residue of nAChRs is Y93 (TyrA) (Figure 1).

	Loop A										Loop B				
	121									130	181				185
Mouse 5-HT$_3$A	W	V	P	D	I	L	I	N	E	F	T	S	W	L	H
Human 5-HT$_3$A	W	V	P	D	I	L	I	N	E	F	T	S	W	L	H
Torpedo nACh α1	W	L	P	D	L	V	L	–	Y	N	G	I	W	T	Y
Human nACh α7	W	K	P	D	I	L	L	–	Y	N	G	S	W	S	Y
Lymnaea AChBP	W	V	P	D	L	A	A	–	Y	N	G	S	W	T	H

Figure 1. Alignment of loops A and B from the mouse and human 5-HT$_3$A subunits, the *Torpedo* nACh α1 subunit, the human nACh α7 subunit, and the *Lymnaea* AChBP. Residues contributing significant interactions through their side chains are highlighted in gray. The numbering is that of the mouse 5-HT$_3$A subunit.

In the present work, we evaluate the role of a second residue on loop A that is very highly conserved across the entire Cys-loop family of neurotransmitter-gated ion channels, D124 of the 5-HT$_3$A receptor. In the nAChR family, the aligning residue, D89, makes a number of hydrogen bonds to loop B, positioning the key TrpB residue.[10−12] This role was suggested by crystal structures of acetylcholine binding proteins (AChBP) and confirmed by extensive mutagenesis studies. In the present work, we find interesting differences between the 5-HT$_3$ receptor and the nAChR with respect to the role of D124. Mutant cycle analyses reveal a collection of interactions in which hydrogen bonds between D124 and loop B serve to position W183, W183 makes the key cation−π interaction, and

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E129 makes binding interactions with serotonin (Figure 2). Triple mutant cycle analysis shows that these three interactions

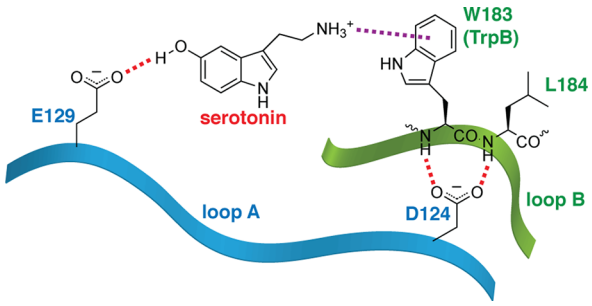

Figure 2. Schematic of the collection of interactions being probed here. Dashed red lines are hydrogen bonds. Purple dashed line is a cation−π interaction.

are tightly coupled to each other, such that disrupting any one interaction destroys the coupling between the other two. In addition, we find that the natural agonist serotonin and the potent orthosteric agonist mCPBG are affected differently by mutations in this region of the receptor.

RESULTS AND DISCUSSION

Serotonin. The 5-HT$_3$A receptor differs from other members of the Cys-loop (pentameric) neurotransmitter-gated ion channel family in that one of the members of the canonical binding site aromatic box (TyrA) is missing (Figure 1). Alignment of loop A across the Cys-loop family is challenging,[9] but our previous functional studies suggested that the side chain of E129 points in toward the agonist binding site, making it the residue that corresponds to TyrA. In particular, we suggested that E129 forms a critical hydrogen bond with the 5-hydroxyl of serotonin. We have now further probed the role of E129 and established a collection of binding interactions that impact receptor function.

Here we propose that another residue on loop A, D124, forms hydrogen bonds with both of the backbone nitrogens flanking the TrpB side chain (Figure 2). Based on the AChBP structures and mutagenesis studies of the nAChR, loop A of the 5-HT$_3$A receptor in the vicinity of D124 lies "behind" loop B, such that the D124 side chain can hydrogen bond to the backbone of loop B in the vicinity of W183. The functional significance of this conserved aspartate has long been appreciated, in that the D-to-N mutation very significantly impedes receptor function in Cys-loop receptors.[11] Not shown in Figure 2 is Ser182, the side chain of which could also contribute to this hydrogen bond network. However, as in studies of the nAChR,[12] we find that the S182A mutation has a negligible effect on receptor function (Table 1), and so we will not further consider hydrogen bonding interactions involving the side chain of S182.

The key potential hydrogen bonding interactions involving D124 are to a pair of backbone NHs on loop B, contributed by W183 and L184. Using nonsense suppression methodology,[13,14] we can perturb the loop B backbone by replacing W183 and L184 with their corresponding α-hydroxy acids (Wah and Lah; ah standing for α-hydroxy) (Figure 3). This subtle mutation deletes the hydrogen bond donor but leaves nearly all other aspects of the receptor intact. The functional effects of these mutations are determined by constructing dose−response curves of the agonist-induced currents (Figure 4).

As shown in Figure 5 and Table 1, both backbone mutations in loop B cause partial loss of function (rise in EC_{50}) with serotonin as agonist. The magnitudes of the perturbations are similar for the two backbone mutations, but they are markedly less than what is seen for the D124N mutation. It is interesting that although these hydrogen bonding interactions were first highlighted in structures of the acetylcholine binding protein (AChBP), comparable backbone mutations of the nAChR did not have strong effects on receptor function.[12]

To establish that the backbone mutations are indeed influencing a hydrogen bond to D124, we performed double mutant cycle analyses[15,16] with the D124N mutation. We consider a $\Delta\Delta G^\circ > 0.65$ kcal/mol (i.e., a 3-fold deviation from additivity) to represent a meaningful interaction energy. For both the W183Wah and the L184Lah mutations, a significant interaction energy is seen when paired with the D124N mutation (Table 2). The magnitude of the interaction is consistent with a hydrogen bond.

Because of the key role of TrpB (W183) in making a cation−π interaction to agonists, it seems reasonable to think of the loop A aspartate as positioning loop B through the hydrogen bonds just probed. However, given our earlier findings[9] of a key role for a second loop A residue (E129), we considered the reverse scenario: that loop B positions two key residues on loop A (Figure 2). If this were so, one expects that disruption of the hydrogen bonding interactions between D124 and W183/L184 would impact E129. We find that this is indeed the case. Both the W183Wah and the L184Lah mutations are coupled via mutant cycle analysis to the E129Q mutation (Table 2), and the magnitude of the coupling is comparable to that measured for couplings to D124N.

These results implied a collection of interactions: the loop B backbone NHs interact with loop A via D124; this in turn positions E129 on loop A properly for interaction with the agonist OH, and the agonist protonated amine makes a cation−π interaction to W183 back on loop B (Figure 2). This suggested experiments that constitute a triple mutant cycle analysis, combining D124N, E129Q, and L184Lah (protein expression for W183Wah mutants was too low to consider triple mutant studies). Such an analysis is best described by a cube, as in Figure 6. The wild type is in the front lower left corner, and the triple mutant is in the back upper right corner. Each face of the cube is a double mutant cycle analysis. Both the front and back faces, for example, depict the D124N/L184Lah double mutant cycle, but in the background of the wild type or the E129Q mutant, respectively (i.e., all four corners of the back face contain the E129Q mutation). As noted above, $\Delta\Delta G$ for the front face is 1.6 kcal/mol. However, $\Delta\Delta G$ for the back face is 0.06 kcal/mol. That is, the coupling between D124N and L184Lah is completely absent if we include the E129Q mutation. The coupling between D124 and L184 (backbone) depends on the presence of the E129 side chain. The difference between the $\Delta\Delta G$ values for the front and back faces produces a $\Delta\Delta\Delta G$, the extent to which the three mutations all influence each other. In the present system, $\Delta\Delta\Delta G$ is 1.5 kcal/mol. The value for $\Delta\Delta\Delta G$ can also be obtained by subtracting $\Delta\Delta G$ values for the left and right faces or the top and bottom faces; the same $\Delta\Delta\Delta G$ value is always obtained. Interestingly, any double mutant cycle analysis that includes the wild-type receptor, the front, left, and bottom faces, shows a significant $\Delta\Delta G$ of ~1.5 kcal/mol. However, the

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Table 1. Agonist Dose−Response Data[a]

agonist	mutation[b]	EC_{50} (μM)	fold shift	Hill	R_{max}	N
5-HT	WT	1.9 ± 0.1		2.7 ± 0.3		7
	W183Wah	27 ± 1	14	2.4 ± 0.3		6
	L184Lah	13 ± 1	6	2.8 ± 0.2		23
	E129Q	88 ± 7	46	1.5 ± 0.1		17
	E129D	2.5 ± 0.3	1	1.6 ± 0.2		7
	S182A	2.6 ± 0.1	1.4	2.3 ± 0.2		7
	D124N	140 ± 10	70	2.6 ± 0.2		6
	E129Q L184Lah	46 ± 10	24	1.5 ± 0.4		9
	E129Q W183Wah	80 ± 10	41	1.8 ± 0.3		7
	D124N L184Lah	61 ± 2	31	2.5 ± 0.1		6
	D124N W183Wah	160 ± 10	85	4.1 ± 0.4		6
	D124N E129Q	490 ± 60	255	3.2 ± 1.0		6
	L184Lah E129Q D124N	230 ± 40	118	3.1 ± 1.3		8
	T6′S	1.8 ± 0.2	0.9	2.3 ± 0.3		6
	T6′S E129Q	6.0 ± 0.5	3	1.4 ± 0.1		6
5-FT	WT	18 ± 1		2.2 ± 0.2	0.60 ± 0.03	6
	W183Wah	[c]				8
	L184Lah	36 ± 4	2	3.2 ± 0.9	0.03 ± 0.01	10
	E129Q	[c]				8
mCPBG	WT	0.61 ± 0.03		2.2 ± 0.2	0.73 ± 0.02	7
	W183Wah	[c]				8
	L184Lah	[c]				8
	E129Q	[c]				8
	E129D	0.12 ± 0.01	0.2	1.1 ± 0.1	0.10 ± 0.01	11
	S182A	0.15 ± 0.01	0.2	3.0 ± 0.4	0.74 ± 0.04	7
	D124N	15 ± 1	24	3.4 ± 0.3	1.09 ± 0.07	6
	D124N L184Lah	55 ± 2	90	3.5 ± 0.3	0.87 ± 0.04	6
	D124N W183Wah	[c]				8
	W183−4-F-W	0.47 ± 0.03	0.8	1.5 ± 0.1	0.44 ± 0.03	6
	W183−4,7-F_2-W	0.34 ± 0.03	0.6	1.4 ± 0.1	[d]	19
	W183−5−F-W	5.8 ± 0.1	10	2.1 ± 0.1	0.11 ± 0.01	8
	W183−5,7-F_2−W	2.8 ± 0.1	5	1.9 ± 0.1	0.38 ± 0.03	8
	W183−5,6,7-F_3-W	9.7 ± 0.3	16	2.4 ± 0.1	0.57 ± 0.03	11
	W183−4,5,6,7-F_4−W	7.3 ± 0.3	12	2.1 ± 0.2	0.59 ± 0.01	8
	W183−5-Br-W	20 ± 2	33	2.5 ± 0.4	0.06 ± 0.01	8
mCPG	WT	0.61 ± 0.03		2.2 ± 0.2	0.73 ± 0.02	7
	E129Q	[c]				8
	E129D	[c]				8
	W183−4-F-W	2.7 ± 0.4	0.8	2.4 ± 0.8	0.23 ± 0.03	7
	W183−4,7-F_2-W	[e]				8
	W183−5-F-W	13 ± 2	4	2.2 ± 0.4	0.09 ± 0.01	7
	W183−5-Br-W	42 ± 3.4	12	1.9 ± 0.3	0.02 ± 0.01	8

[a]All values are reported as mean ± SEM. Fold shift >1 indicates loss of function; fold shift < 1 indicates gain of function. [b]Xah = α-hydroxy X. [c]No response (I_{max} < 20 nA). [d]Not determined. [e]Small response (I_{max} < 100 nA).

remaining three faces, all of which have one of the three mutations present in all four corners, show $\Delta\Delta G$ values of ∼0. Having any one of the three mutations completely decouples the other two, suggesting a very tight interaction among the three.

If the proposed model of E129 making a hydrogen bond to the OH of serotonin is correct, we expect that mutations that influence E129 would impact this interaction. We previously showed that 5-FT (Figure 3) has a potency ∼10-fold lower than 5-HT and acts as a partial agonist with a relative efficacy (R_{max}) of 0.64.[17] Assuming 5-FT binds in essentially the same manner as serotonin, the F of 5-FT would be near the side chain of E129 but cannot make a hydrogen bond. The high relative efficacy of 5-FT compared with that of tryptamine (serotonin without the OH; R_{max} = 0.15) suggests that 5-FT retains a steric or electrostatic interaction at the 5 position that contributes to receptor function. Interestingly, when we introduce the E129Q mutation, 5-FT becomes an antagonist, with an IC_{50} value comparable to its EC_{50} in the wild-type receptor (Table 3). As expected, given their strong coupling to E129, the W183Wah and L184Lah backbone mutations behave similarly, strongly disrupting the ability of 5-FT to gate the receptor. W183Wah converts 5-FT to a weak antagonist, while the efficacy of 5-FT relative to serotonin decreases to three percent in the background of L184Lah. This weak agonism allows consideration of serotonin/5-FT as one component of a double mutant cycle analysis. When the agonist pair is coupled to the L184Lah backbone mutation, the $\Delta\Delta G$ value (0.73 kcal/mol) is smaller than others we have seen but clearly

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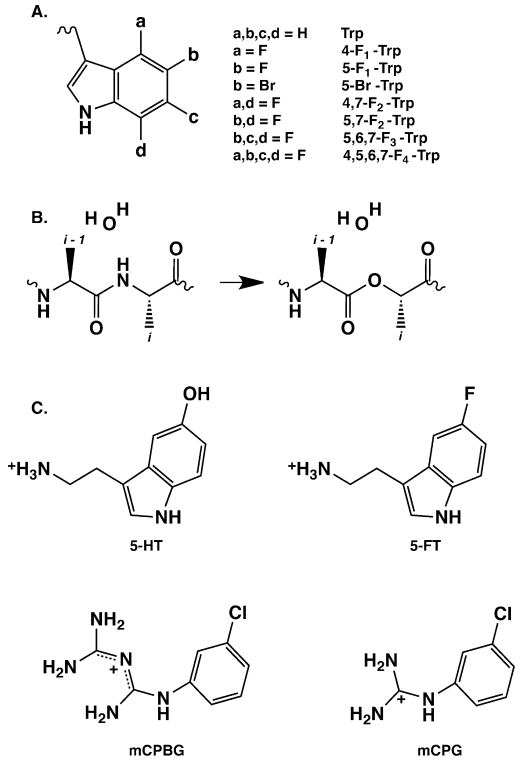

Figure 3. (A) Structures of unnatural amino acids considered here. Unless specified, a, b, c, and d are H. (B) The α-hydroxy acid strategy for probing backbone hydrogen bonding interactions. (C) Structures of agonists considered here.

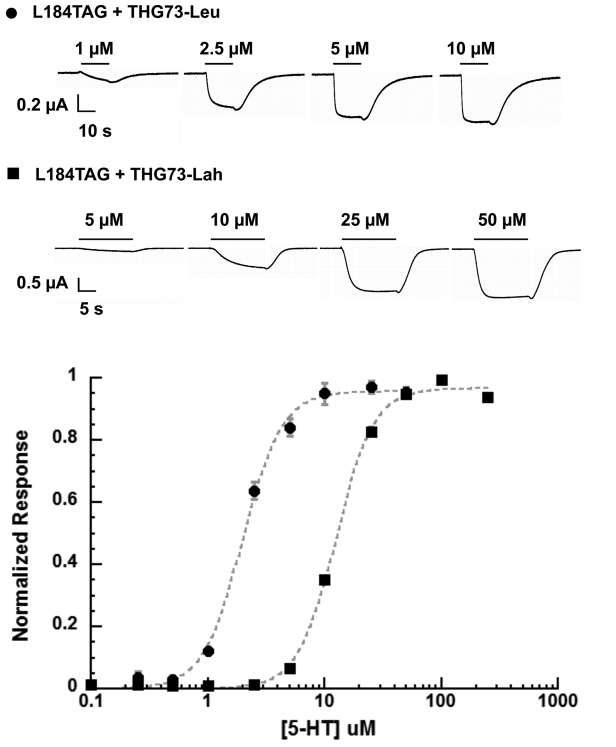

Figure 4. Examples of responses for 5-HT on receptors in which a nonsense mutation (TAG) at L184 is suppressed by THG73 tRNA bearing either leucine (wild-type recovery) or α-hydroxy leucine expressed in *Xenopus* oocytes and their corresponding concentration–response curves. Circles correspond to L184TAG + THG73-Leu; squares correspond to L184TAG + THG73-Lah. Each data point represents the mean ± SEM (n = 7–23).

meaningful, bolstering the interpretation that the backbone NHs and E129 are linked.

In the model that we are proposing (Figure 2), two of the interactions considered, the cation–π interaction to TrpB and the hydrogen bond between E129 and the OH of serotonin, involve direct binding between the receptor and the drug. Two others, the hydrogen bonds to the backbone of loop B, occur within the receptor itself. It may be that these various interactions impact different aspects of the receptor activation process.

The metric we use to evaluate receptor function is EC_{50}, the effective concentration of agonist needed to induce half-maximal response. This is a functional measure of agonist potency. The actual process of activating the 5-HT_3A receptor and related molecules is complex, involving multiple equilibria reflecting drug binding to and coming off the receptor, conformational changes of the protein, and "gating" equilibria between the open and closed states of the channel. It is important to acknowledge the ambiguity that a change in EC_{50} could reflect a change in "binding" or a change in "gating". Certainly there is value in knowing which step(s) in the gating process is impacted by a particular mutation, but this requires much more detailed studies, typically at the single channel level; such studies are further complicated in the present case by the low single-channel conductance of the 5-HT_3A receptor. An alternative strategy would be to determine K_i values for various ligands at mutant receptors. The standard strategy for 5-HT_3 receptors is displacement of tritiated granisetron. However, it has been reported that various mutants at E129, including E129Q, show no binding of granisetron.[18] A further complication with the determination of binding constants is the large amount of receptor required, quantities greater than what can typically be produced with unnatural amino acid mutagenesis. In light of these limitations, we do not believe binding studies would be feasible on the most interesting of the mutants studied here.

An alternative way to evaluate the role a particular residue plays is through employing a different type of mutant cycle analysis. In previous studies of the nAChR, we have often introduced a mutation in the transmembrane domain, quite far from the agonist binding site, that facilitates gating and thereby lowers EC_{50} in a general way.[19,20] Typically, such transmembrane mutations show simple additivity when paired with a second mutation in the agonist binding site ($\Delta\Delta G$ = 0). However, meaningful coupling between the two sites can be observed when the agonist binding site mutation significantly impacts gating. We considered a similar strategy here, using a mutation in M2 termed T6′S,[21] where 6′ denotes the sixth amino acid from the cytoplasmic end of the transmembrane helix. As noted earlier, the E129Q mutation is strongly perturbing, producing a high EC_{50} of 88 μM (46-fold shift from wild type) for serotonin (Table 1). With the addition of the T6′S mutation, EC_{50} drops 15-fold, to 6.0 μM, following a pattern we have seen with other mutations of this sort. However, the same effect is not seen in the otherwise wild-type receptor; the T6′S single mutant shows an EC_{50} of 1.8 μM, indistinguishable from wild-type. Of course, this means that the T6′S and E129Q mutations are coupled, and a mutant cycle analysis gives a large $\Delta\Delta G$ of 1.6 kcal/mol (Table 2). Since the T6′S mutation presumably affects channel gating, the coupling seen here suggests that the proposed hydrogen bond between

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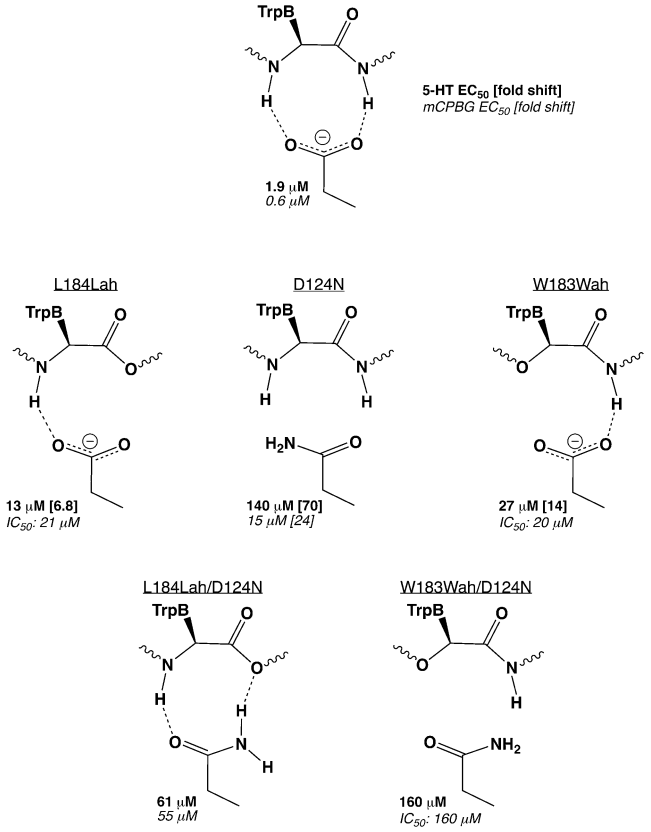

Figure 5. Results of select mutagenesis studies.

Table 2. Serotonin Mutant Cycle Analyses

mutant pair[a]	background	Ω^b	$\Delta\Delta G°$ (kcal/mol)
W183Wah, D124N		0.080	1.5
W183Wah, E129Q		0.064	1.6
L184Lah, D124N		0.064	1.6
L184Lah, D124N	E129Q	0.90	0.062
L184Lah, E129Q		0.076	1.5
L184Lah, E129Q	D124N	0.93	0.043
E129Q, D124N		0.076	1.5
E129Q, D124N	L184Lah	0.94	0.037
L184Lah, 5-HT/5-FT		0.29	0.73
E129Q, T6′S		0.072	1.6

[a]Xah = α-hydroxy X. [b]Coupling coefficient.

E129 and the 5-hydroxyl of serotonin contributes to the initiation of channel gating.

mCPBG. In addition to the endogenous ligand serotonin, the 5-HT_3A receptor is capable of being gated by many structurally diverse molecules, including the potent orthosteric agonist mCPBG. The biguanide mCPBG (Figure 3)[22] is an agonist at 5-HT_3 receptors with a potency comparable to that of serotonin and a relative efficacy of 0.74. The structures of 5-HT_3 and mCPBG are different enough that is seemed possible that the two might interact differently with the receptor. Along with a clearly different shape and size (Figure 7), mCPBG displays a weak and diffuse positive electrostatic potential across its biguanide moiety compared with the primary amine of serotonin. The chlorine atom of mCPBG may provide some steric and electronegative interactions of the sort shown to be important for 5-FT gating but without the hydrogen bonding ability of the serotonin OH. We have probed all the mutations discussed above using mCPBG as the agonist, with intriguing results. As with serotonin, the D124N mutation produces marked loss of function.

A surprising result is seen with both of the backbone mutations: when either W183Wah or L184Lah is introduced, mCPBG becomes an *antagonist*, with a comparable IC_{50} for both mutants (Table 3). Remarkably, restoring the hydrogen bond between L184 and D124 by pairing a D124N mutation with a backbone ester at L184 rescues the ability of mCPBG to gate the receptor, with significant loss-of-function but a relative efficacy of 0.87 (Figure 5). The W183Lah/D124N receptor still shows mCPBG as an antagonist, with a further elevated IC_{50}, suggesting that the two backbone mutants may assume different geometries. We cannot do mutant cycle analyses on these systems, because one cannot meaningfully mix EC_{50} and IC_{50} values. The functional necessity of the hydrogen bonds linking loops A and B suggests that proper positioning of E129 is critical for mCPBG channel gating.

There are two other important ways in which mCPBG responds to mutations in a qualitatively distinct way from serotonin. First, the single mutation E129Q also converts mCPBG into an antagonist. Second, activation or block by mCPBG is not affected by fluorination of the W183 side chain, although serotonin and many agonists at other Cys-loop receptors are affected by such fluorination.[5] Substituting W183 with 5-F-Trp does produce a 10-fold increase in EC_{50}, but further fluorination does not produce the progressive increases in EC_{50} that are indicative of a cation−π interaction. This suggested that the 5-F-Trp effect could reflect a steric interaction, so we incorporated 4-F-Trp (Figure 3). This should produce an equivalent response if we are probing a cation−π interaction but a different response if steric interactions are important. The EC_{50} value for 4-F-Trp was close to wild-type. Increased fluorination, avoiding the 5 position, also resulted in near wild-type behavior. Greatly increasing the steric bulk at the 5 position via 5-Br-Trp led to a larger increase in EC_{50}, further supporting the idea of a steric interaction. This lack of fluorination effect could be due to a slight positioning difference, or it could be that the positive charge of mCPBG is so diffuse that it cannot pick up an energetically significant electrostatic interaction with the side chain of W183.

mCPG. The guanide analogue of mCPBG, termed mCPG (Figure 3, 7), is also an agonist at 5-HT_3 receptors with an efficacy relative to serotonin of 0.31. Compared with mCPBG, the smaller mCPG contains a less delocalized positive electrostatic potential that would seemingly be more capable of forming a cation−π interaction. As with the biguanide however, mCPG shows wild-type behavior with the 4-F-Trp mutant at W183 and increasing losses of function as increasing steric bulk is introduced at the 5 position.

As with the biguanide, mCPG also forms a crucial interaction at E129. Mutation to glutamine results in the conversion of mCPG to an antagonist with an IC_{50} comparable to that of mCPBG. mCPG behaves uniquely in one regard however (Table 3). E129D, which is only mildly disruptive for both serotonin and mCPBG, converts mCPG to an antagonist with an IC_{50} in the low nanomolar range, or ∼100-fold stronger inhibition than that observed for any other converted agonist.

■ CONCLUSIONS

Together these results suggest an interesting cycle of interactions that mutually reinforce each other. The essential

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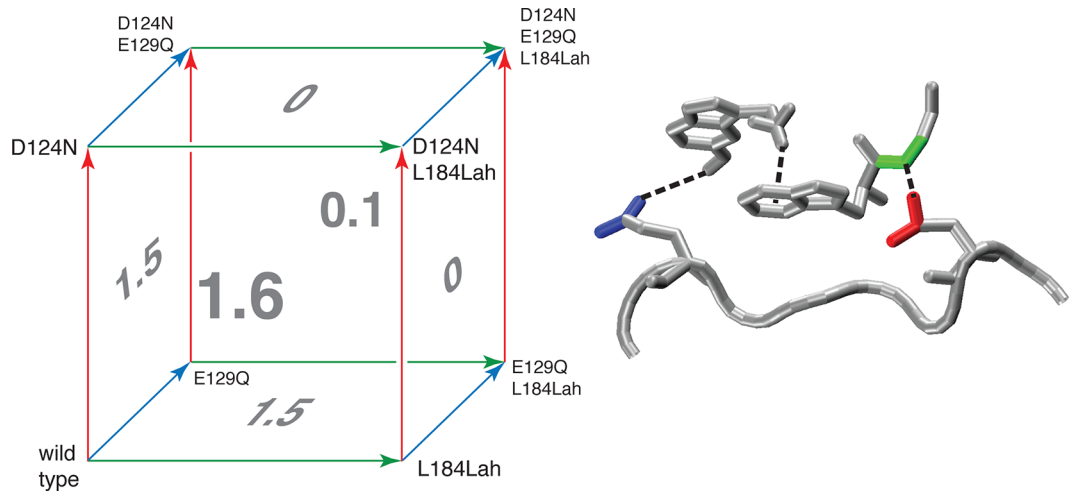

Figure 6. Triple mutant cycle analysis. ΔΔ*G* values (kcal/mol) are given on each face of the cube. ΔΔΔ*G* for the diagonal from lower left front to upper right back is 1.5 kcal/mol. The structural image is a simple docking of 5-HT into a previously described[9] homology model of the receptor and is for illustrative purposes only. See text for a full description and analysis.

Table 3. Dose−Response Data for Mutations in Which the Ligand Is Converted to an Antagonist[a]

mutation[b]	antagonist	IC_{50} (μM)	Hill	*N*
L184Lah	mCPBG	21 ± 1	−2.1 ± 0.2	12
W183Wah	mCPBG	20 ± 1	−1.8 ± 0.1	7
	5-FT	140 ± 20	−1.6 ± 0.3	11
W183Wah D124N	mCPBG	160 ± 10	−2.6 ± 0.3	7
E129Q	mCPBG	0.28 ± 0.03	−1.6 ± 0.1	6
	mCPG	0.32 ± 0.08	−1.2 ± 0.3	7
	5-FT	8.6 ± 2.7	−0.8 ± 0.2	7
E129D	mCPG	0.0094 ± 0.0024	−0.9 ± 0.1	11

[a]All values are reported as mean and SEM. [b]Xah = α-hydroxy X.

role of TrpB for the natural agonist serotonin is clear, making a cation−π interaction to the positive charge of the neurotransmitter. Loop A residue D124 makes two hydrogen bonds to loop B that no doubt serve to position TrpB, an effect that has been seen in the nAChR. Also on loop A, E129 interacts strongly with serotonin, and we propose that this interaction is a hydrogen bond to the OH of the agonist. The interconnectedness of these interactions suggested in Figure 2 is supported by mutant cycle analyses. Several pairwise interactions are significant, and the triple mutant cycle analysis of Figure 6 is especially compelling. In particular, we find that mutating any one of the trio of residues D124, E129, or L184 (backbone) decouples the other two from each other. This suggests that this cyclic array acts as a single unit, with an important functional role in the 5-HT_3 receptor.

We have also found that the structurally distinct agonist mCPBG displays some differences from serotonin. In particular, the cation−π interaction to TrpB that is seen for serotonin and many other agonists at Cys-loop receptors is not present for mCPBG activating the 5-HT_3A receptor.

METHODS

Mutagenesis and Preparation of cRNA and Oocytes. Mutant 5-HT_3A receptor subunits, within the complete coding sequence for the mouse 5-HT_3A receptor subunit (accession number Q6J1J7), were cloned into pcDNA3.1 (Invitrogen, Abingdon, U.K.). Mutagenesis reactions were performed using the QuikChange mutagenesis kit (Stratagene) and confirmed by DNA sequencing. For unnatural mutagenesis, a stop codon, TAG, was made at the site of interest. Harvested stage V−VI *Xenopus* oocytes were washed in four changes of Ca-free OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), defolliculated in 1 mg/mL collagenase for approximately 1 h, washed again in four changes of Ca-free OR2 and transferred to ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 0.28 mg/mL pyruvate, 0.05 mg/mL Gentamicin, and 0.12 mg/mL theophylline. Oocytes were injected with 5−25 ng of mRNA produced by *in vitro* transcription using the mMESSAGE mMACHINE kit (Ambion, Austin, Texas, USA) from cDNA subcloned into pGEMHE as

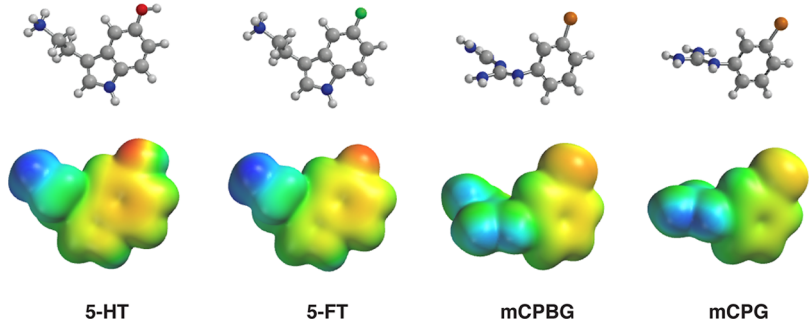

Figure 7. Structures and electrostatic potential surfaces for agonists considered here. Structures and surfaces were generated at the HF 3-21G* level. Electrostatic potentials are plotted with blue most positive and red least positive over a range from 0 to +750 kJ/mol. For the individual molecules, the electrostatic potentials span the following ranges: 5-HT, 13−733; 5-FT, 47−745; mCPBG, 116−636; mCPG, 152−698.

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previously described.[13] For unnatural amino acid experiments, a 1:1 mixture of mRNA and THG73 tRNA charged with an unnatural amino acid were injected (5−25 ng each). Electrophysiological measurements were performed after incubation for 24−72 h postinjection at 18 °C. Wild-type recovery experiments (injection of tRNA appended to the natural amino acid) were performed to evaluate the fidelity of the unnatural suppression experiments. The 76-mer THG73 was also injected with mRNA as a control.

Synthesis of tRNA and dCA Amino Acids. This was as described previously.[13] Briefly, unnatural amino acids were chemically synthesized as nitroveratryloxycarbonyl (NVOC)-protected cyanomethyl esters and coupled to the dinucleotide dCA, which was then enzymatically ligated to 74-mer THG73 $tRNA_{CUA}$. Immediately prior to coinjection with cRNA, aminoacyl tRNA was deprotected by photolysis. Typically 5−10 ng of total cRNA was injected with 25−50 ng of tRNA-aa in a total volume of 50 nL. For a control, cRNA was injected with THG 76-mer tRNA (no unnatural amino acid attached).

Characterization of Mutant Receptors. Agonist-induced currents were recorded at 22−25 °C from individual oocytes using the OpusXpress system (Molecular Devices Axon Instruments, Union City, CA). 5-HT, *m*-chlorophenylbiguanide (mCPBG), *m*-chlorophenylguanide (mCPG), and 5-fluorotryptamine (5-FT) (Sigma) were stored as 25 mM aliquots at −20 °C, diluted in Ca-free ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) and delivered to cells via the automated perfusion system of the OpusXpress. Glass microelectrodes were backfilled with 3 M KCl and had a resistance of ∼1 MΩ. The holding potential was −60 mV. Agonist doses in Ca^{2+}-free ND96 were applied for 15 s followed by a 116 s wash with the running buffer. Dose−response data were obtained for ≥8 agonist concentrations on ≥6 cells. To determine EC_{50} values, concentration−response data were fitted to the four-parameter logistic equation, $I = I_{max}/[1 + (EC_{50}/[A])^{nH}]$where I_{max} is the maximal response plateau, [A] is the log concentration of agonist, and nH is the Hill coefficient, using KaleidaGraph v3.6 software (Synergy Software, Reading, PA). A detailed error analysis of nonsense suppression experiments shows that data are reproducible to ±50% in EC_{50}.[23] Relative efficacies (ε) of the partial agonists mCPBG, mCPG, and 5-fluorotryptamine (5-FT) are reported as $\varepsilon = I_{max\text{-}drug}/I_{max\text{-}5\text{-}HT}$. Efficacy was calculated for individual cells and then averaged and reported as mean ± SEM.

The coupling coefficient, Ω, is defined as

$$\frac{EC_{50}(\text{wt, wt})EC_{50}(\text{mut, mut})}{EC_{50}(\text{wt, mut})EC_{50}(\text{mut, wt})}$$

and the coupling energy of the double mutation, $\Delta\Delta G = RT \ln(\Omega)$.

For receptor antagonist experiments, antagonist doses in Ca^{2+}-free ND96 were applied to the oocyte for 15 s and incubated for a further 45 s. An identical dose of antagonist was then applied in a concentration of serotonin equal to 2-fold the mutant EC_{50} for 15 s before a 124 s wash with the running buffer. To ensure that current decreases result from antagonist alone, consistency of cell responses was determined by applying serotonin of concentration equal to 2-fold mutant EC_{50} for 15 s followed by a 124 s wash with the running buffer prior to the first antagonist dose and following a 5 min washout with running buffer after every fourth subsequent antagonist dose. Dose−response data were obtained for ≥8 agonist concentrations on ≥6 cells. To determine IC_{50} values, concentration−response data were fitted to the four-parameter logistic equation, $I = I_{max}/[1 + (IC_{50}/[A])^{nH}]$where I_{max} is the maximal response plateau, [A] is the log concentration of agonist, and nH is the Hill coefficient, using KaleidaGraph v4.1.2 software (Synergy Software, Reading, PA).

Computation. Calculations were performed with the program SPARTAN.[24]

AUTHOR INFORMATION

Corresponding Author

*Mailing address: California Institute of Technology, 164-30, Pasadena, CA 91125. E-mail: dadougherty@caltech.edu. Telephone: 626-395-6089. Fax: 626-564-9297.

Author Contributions

T.F.M., K.S.B., H.A.L., and D.A.D. contributed to research design. T.F.M. and K.S.B. conducted experiments. T.F.M., K.S.B., H.A.L., and D.A.D. performed data analysis. T.F.M., K.S.B., H.A.L., and D.A.D. wrote the manuscript.

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Notes

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ABBREVIATIONS

5-HT, 5-hydroxytryptamine, serotonin; 5-FT, 5-fluorotryptamine; mCPBG, *m*-chlorophenylbiguanide; mCPG, *m*-chlorophenylguanide; 5-HT_3A, serotonin receptor type 3A; nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein

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