

Varenicline Interactions at the 5-HT₃ Receptor Ligand Binding Site are Revealed by 5-HTBP

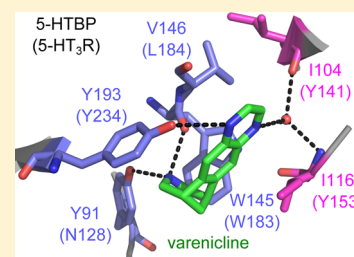
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S Supporting Information

ABSTRACT: Cys-loop receptors are the site of action of many therapeutic drugs. One of these is the smoking cessation agent varenicline, which has its major therapeutic effects at nicotinic acetylcholine (nACh) receptors but also acts at 5-HT₃ receptors. Here, we report the X-ray crystal structure of the 5-HT binding protein (5-HTBP) in complex with varenicline, and test the predicted interactions by probing the potency of varenicline in a range of mutant 5-HT₃ receptors expressed in HEK293 cells and *Xenopus* oocytes. The structure reveals a range of interactions between varenicline and 5-HTBP. We identified residues within 5 Å of varenicline and substituted the equivalent residues in the 5-HT₃ receptor with Ala or a residue with similar chemical properties. Functional characterization of these mutant 5-HT₃ receptors, using a fluorescent membrane potential dye in HEK cells and voltage clamp in oocytes, supports interactions between varenicline and the receptor that are similar to those in 5-HTBP. The structure also revealed C-loop closure that was less than in the 5-HT-bound 5-HTBP, and hydrogen bonding between varenicline and the complementary face of the binding pocket via a water molecule, which are characteristics consistent with partial agonist behavior of varenicline in the 5-HT₃ receptor. Together, these data reveal detailed insights into the molecular interaction of varenicline in the 5-HT₃ receptor.



KEYWORDS: Ligand-gated ion channel, Cys-loop receptor, serotonin receptor

The 5-HT₃ receptors belong to the Cys-loop family of pentameric ligand-gated ion channels, a family that also includes nicotinic acetylcholine (nACh), GABA, and glycine receptors.¹ They play important roles in fast neurotransmission in the central and peripheral nervous systems. There are five 5-HT₃ receptor subtypes (5-HT_{3A}–5-HT_{3E}). Subunits of 5-HT_{3A} can assemble to form homopentamers, but the other subtypes must coassemble with 5-HT_{3A} subunits to form functional receptors. However, it is as yet unclear what effect subunits 5-HT_{3C}–5-HT_{3E} have on the receptor function.^{2,3}

Varenicline is a high affinity partial agonist of the $\alpha 4\beta 2$ nACh receptor in clinical use for smoking cessation.⁴ It is also a full agonist at the $\alpha 7$ nACh receptor.⁵ There is considerable overlap between compounds that act at $\alpha 7$ nACh and 5-HT₃ receptors,⁶ and recently, we have shown that varenicline is also a high affinity agonist at the human 5-HT₃ receptor.⁷ 5-HT₃ receptors are located in the chemoreceptor trigger zone in the area postrema and in the gastrointestinal tract, where they have roles in regulating gut motility and the emesis reflex.⁸ Antagonists of 5-HT₃ receptors, such as ondansetron and palonosetron, are in clinical use as antiemetics to treat postoperative nausea and vomiting and chemotherapy-induced nausea and vomiting.⁹ Nausea is a common adverse effect reported from patients receiving varenicline,⁴ and it is likely that this is due to its actions at the 5-HT₃ receptor. The design of improved smoking cessation drugs that do not have such

cross-reactivity at the 5-HT₃ receptor would clearly be of benefit.

For this aim to be achieved, further information is needed about the structure–activity relationships of varenicline binding to different receptors. Here, we present the structure of varenicline bound to 5-HTBP, an acetylcholine binding protein (AChBP) from *Aplysia californica* (Ac) engineered to bind 5-HT with high affinity.¹⁰ This protein carries the mutations S92E, V140L, K141T, and Q55R (A1B2D1_R) and displays affinities for the 5-HT₃ receptor antagonist granisetron and for 5-HT that are 21- and 4.3-fold higher than for native AChBP, respectively. We have also performed a thorough mutagenesis study on the human 5-HT₃ receptor of residues predicted to be near the ligand-binding site, characterizing these mutants both in HEK293 cells using a fluorescent membrane potential dye and in oocytes using two-electrode voltage clamp electrophysiology. The data suggest that 5-HTBP is a good structural model for examining ligand binding interactions, and functional experiments on the 5-HT₃ receptors yield additional information on binding modes that cannot readily be ascertained from structural information.

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Table 1. Concentration Response Parameters of 5-HT Obtained from HEK Cells

loop	mutant	5-HT pEC ₅₀ ^a (M)	5-HT EC ₅₀ (μM)	n
A	WT	6.51 ± 0.03	0.31	5
	N128	N128A	5.47 ± 0.10 ^b	3.4
		N128Q	4.53 ± 0.02 ^b	29
B	W183	W183A	3.88 ± 0.05 ^b	4
		W183Y	4.60 ± 0.03 ^b	25
B	L184	L184A	NR ^c	6
		L184I	5.56 ± 0.05 ^b	2.8
C	F226	F226A	4.18 ± 0.05 ^b	6
		F226Y	5.72 ± 0.06 ^b	1.9
C	M228	M228A	5.03 ± 0.04 ^b	4
		M228C	NR ^c	NR ^c
C	E229	E229A	5.63 ± 0.04 ^b	2.3
		E229D	6.58 ± 0.05	0.27
C	Y234	Y234A	NR ^c	6
		Y234F	5.89 ± 0.03 ^b	1.3
		Y234S	NR ^c	NR ^c
D	W90	W90A	NR ^c	6
		W90Y	5.66 ± 0.05 ^b	2.2
D	R92	R92A	6.06 ± 0.04 ^b	0.87
		R92Q	4.82 ± 0.04 ^b	15
		Y94A	7.24 ± 0.03 ^b	0.058
D	Y94	Y94F	6.84 ± 0.04	0.14
		Y94S	7.03 ± 0.06 ^b	0.093
		S114A	6.31 ± 0.05	0.48
E	S114	S114T	5.87 ± 0.27 ^b	1.4
	Y141	Y141A	NR ^c	NR ^c
		Y141F	6.83 ± 0.03	0.15
E	Y143	Y143A	NR ^c	NR ^c
		Y143F	4.98 ± 0.09 ^b	11
		Y143S	4.35 ± 0.03 ^b	45
E	Q151	Q151A	6.13 ± 0.06	0.75
		Q151N	6.34 ± 0.03	0.45
		Y153A	4.73 ± 0.03 ^b	19
E	Y153	Y153F	5.54 ± 0.03 ^b	2.9
		Y153S	5.32 ± 0.06 ^b	4.8

^aData = mean ± SEM. ^bSignificantly different ($p < 0.05$) than that of WT 5-HT_{3A} receptors. ^cNR = no response at 100 mM 5-HT.

pine nitrogen of varenicline. The same interaction in 5-HT₃ receptors is supported by a lack of function in 5-HT₃W183A receptors and is also consistent with data from varenicline studies at the α4β2 nACh receptor,¹⁶ although varenicline does not participate in a cation-π interaction with the TrpB residue in the α7 nACh receptor.¹⁷ Another important loop B residue is L184. Substitution of this residue to Ile resulted in an ~10 fold increase in EC₅₀ with receptors expressed in HEK cells, but no response was observed when we substituted this residue with Ala. We did, however, observe responses in oocytes (Tables 3 and 4), which revealed a 46-fold increase in EC₅₀ and a decrease in $R_{\max}/R_{\max,5-HT}$ from 0.73 to 0.26 compared to those of WT receptors (Figure 3). A previous study has shown that a backbone L184 mutation causes partial loss of function and converts the partial agonist mCPBG into an antagonist.¹⁸ There is also increasing evidence that loop B is important for the structure rather than the function of the receptor.¹⁹ We therefore suggest that the role of L184 may be to define the correct structure of the binding site.

The data show that the aromatic rings of the loop C residues F226 and Y234 are important. Conservation of aromaticity in

Table 2. Varenicline Concentration Response Parameters Obtained from HEK Cells

loop	mutant		varenicline pEC ₅₀ ^a (M)	varenicline EC ₅₀ (μM)	n
A	WT		6.38 ± 0.02	0.42	5
	N128	N128A	5.36 ± 0.05 ^b	4.4	3
		N128Q	4.58 ± 0.03 ^b	26	3
B	W183	W183A	NR ^c	NR ^c	6
		W183Y	4.43 ± 0.04 ^b	38	5
B	L184	L184A	NR ^c	NR ^c	6
		L184I	5.45 ± 0.04 ^b	3.6	5
C	F226	F226A	4.36 ± 0.12 ^b	44	3
		F226Y	5.99 ± 0.05 ^b	1.0	3
C	M228	M228A	4.53 ± 0.04 ^b	30	3
		M228C	NR ^c	NR ^c	6
C	E229	E229A	5.38 ± 0.18 ^b	4.2	3
		E229D	5.98 ± 0.05 ^b	1.1	3
C	Y234	Y234A	NR ^c	NR ^c	6
		Y234F	5.96 ± 0.04 ^b	1.1	4
		Y234S	NR ^c	NR ^c	6
D	W90	W90A	NR ^c	NR ^c	6
		W90Y	5.54 ± 0.03 ^b	2.9	4
D	R92	R92A	6.49 ± 0.06	0.39	4
		R92Q	5.19 ± 0.03 ^b	6.4	4
D	Y94	Y94A	6.89 ± 0.02 ^b	0.13	5
		Y94F	6.43 ± 0.02	0.37	5
		Y94S	6.82 ± 0.04 ^b	0.15	6
	S114	S114A	6.16 ± 0.05	0.95	3
E		S114T	5.36 ± 0.05 ^b	4.3	4
	Y141	Y141A	NR ^c	NR ^c	6
		Y141F	6.56 ± 0.07	0.28	4
E	Y143	Y143A	NR ^c	NR ^c	6
		Y143F	SR ^d	SR ^d	6
		Y143S	SR ^d	SR ^d	6
E	Q151	Q151A	6.22 ± 0.05	0.61	3
		Q151N	5.67 ± 0.06 ^b	2.1	4
E	Y153	Y153A	5.03 ± 0.04 ^b	9.4	3
		Y153F	5.50 ± 0.06 ^b	3.2	3
		Y153S	5.38 ± 0.04 ^b	4.2	3

^aData = mean ± SEM. ^bSignificantly different ($p < 0.05$) than that of WT 5-HT_{3A} receptors. ^cNR = no response at 100 mM varenicline ^dSR = responses too small to obtain parameters.

the 5-HT₃F226Y receptor had little effect on the EC₅₀, whereas a non-aromatic residue ablated agonist-gated currents, suggesting a critical hydrophobic interaction. The important role of Y234 has been shown in many studies, and the data were similar here. Both an aromatic and a hydroxyl group play a role: removal of the former in 5-HT₃Y234A receptors ablated function, and removal of the latter in 5-HT₃Y234F receptors increased the EC₅₀. In 5-HTBP, the equivalent residue forms a hydrogen bond with varenicline, and we propose a similar role here. In the α7 nACh receptor, however, the residue equivalent to Y234 (TyrC2) is not important for binding varenicline (although it is involved in the binding of ACh and epibatidine).^{17,20} In the previously reported varenicline-bound AChBP structures (4AFG¹¹ and 4AFT¹²), varenicline is further away from this residue; a H bond is only predicted in one of the five binding sites for Ac-AChBP (4AFT) and may occur via a bridging water molecule in one of the *Capitella telata*-AChBP sites (Figure S2, Supporting Information). Thus, it seems that varenicline may have different interactions with different receptors.

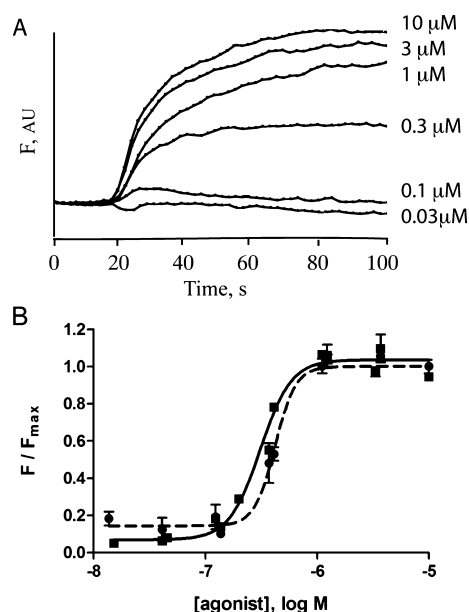


Figure 2. HEK cell data. (A) Typical traces from HEK cells transfected with WT 5-HT₃ receptor cDNA, loaded with membrane potential dye, and stimulated at 20 s with various concentrations of varenicline. F = arbitrary fluorescent units. (B) Concentration–response curves constructed from FlexStation responses to 5-HT (squares, filled line) and varenicline (circles, dashed line). Data = mean \pm SEM, n = 4.

Table 3. Concentration Response Parameters of 5-HT Obtained from Oocytes

mutant	5-HT pEC ₅₀ ^a (M)	5-HT EC ₅₀ (μM)	n	5-HT fold change (EC _{50,mut} /EC _{50,WT})
WT	5.65 \pm 0.03	2.2	5	
L184A	4.12 \pm 0.03 ^b	76	5	40
W90Y	4.97 \pm 0.04 ^b	11	3	5
Y143F	4.78 \pm 0.10 ^b	17	4	7.7
Y143S	4.22 \pm 0.09 ^b	60	4	27
Y153F	4.66 \pm 0.04 ^b	22	4	10

^aData = mean \pm SEM. ^bSignificantly different (p < 0.05) than that of WT 5-HT_{3A} receptors.

Varenicline Interactions at the Complementary Binding Face. The structure revealed hydrogen bonds via a water molecule to the backbone OH of I104 and NH of I116 (loop E), and Y53 forms part of the aromatic box that surrounds varenicline. To probe interactions with these and other residues in the 5-HT₃ receptor, we mutated W90, R92, and Y94 in loop D, S114 (not in a binding loop), and Y141, Y143, Q151, and Y153 in loop E, all of which are within 5 Å of varenicline.

Of the loop D residues, W90 (equivalent to 5-HTBP Y53) appears to be the most important for functional responses to 5-

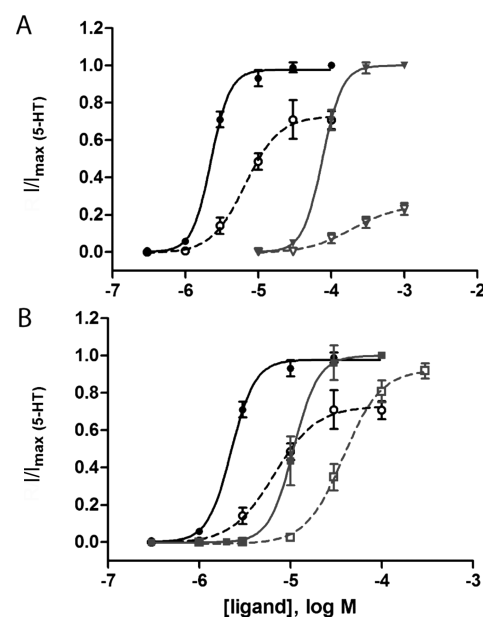


Figure 3. Concentration response curves for WT and mutant 5-HT₃ receptors expressed in oocytes. Concentration response curves for (A) 5-HT_{3A} L184A and (B) 5-HT_{3A} W90Y mutant receptors (gray lines) compared to WT 5-HT_{3A} receptors (black lines) for 5-HT (solid lines) and varenicline (dashed lines) reveal differences in EC₅₀ and R_{\max} / $R_{\max,5-HT}$. Data = mean \pm SEM, n = 3–6.

HT and varenicline. W90 has been previously investigated in mouse 5-HT₃ receptors, and its aromatic group is essential for binding.²¹ Here, we show that substitution with Tyr, which has only a small effect on EC₅₀, significantly increases the efficacy of varenicline, making it a full agonist (Figure 3, Table 4). In the $\alpha 4\beta 2$ nACh receptor, mutation of the equivalent Trp residue also increased efficacy, in this case from 15 to 125%.¹¹ A different effect was observed in the $\alpha 7$ nACh receptor, where the equivalent mutation causes the EC₅₀ of varenicline to increase much more than it does for ACh.¹⁷ These agonist-specific effects at multiple receptors indicate that this residue may be an important determinant of affinity and efficacy across the superfamily, even though it may interact differently with different ligands.

Substitution of the loop E residues Y141, Y143, and Y153 causes large changes in EC₅₀ or even ablates function. Of these residues, Y143 appears to play the biggest role as mutating this residue in the 5-HT₃ receptor has large effects on both the EC₅₀ and the R_{\max} of varenicline, which is only 13% in Y143F mutant receptors. Previously, we suggested that a hydrogen bond formed by this residue was essential for receptor gating.²² With the benefit of the recent high-resolution structure of the 5-HT₃ receptor,¹³ we can see that the Y143 hydroxyl does indeed form

Table 4. Varenicline Concentration Response Parameters Obtained from Oocytes

mutant	varenicline pEC ₅₀ ^a (M)	varenicline EC ₅₀ (μM)	varenicline fold change (EC _{50,mut} /EC _{50,WT})	varenicline efficacy (R_{\max} / $R_{\max,5-HT}$)
WT	5.18 \pm 0.08	6.6		0.73 \pm 0.05
L184A	3.71 \pm 0.03 ^b	193	46	0.26 \pm 0.03 ^b
W90Y	4.42 \pm 0.05 ^b	38	5.8	0.92 \pm 0.05 ^b
Y143F	4.01 \pm 0.07 ^b	97	15	0.13 \pm 0.01 ^b
Y143S	3.85 \pm 0.10 ^b	142	22	0.33 \pm 0.04 ^b
Y153F	4.55 \pm 0.05 ^b	28	4.2	0.76 \pm 0.04

^aData = mean \pm SEM. ^bSignificantly different (p < 0.05) than that of WT 5-HT_{3A} receptors.

hydrogen bonds to D189 and T186 backbone atoms across the binding interface (Figure 4). Because both of these residues

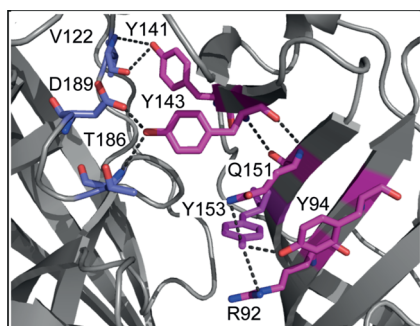


Figure 4. The 5-HT₃ receptor loop E region. Structural data from the mouse 5-HT₃A receptor structure (PDB: 4PIR) reveal a network of hydrogen bonds involving the loop E aromatic residues Y141, Y143, and Y153. Functional data show the importance of these residues for proper functioning of the receptor.

have been previously identified as important for receptor function,^{23,24} it seems likely that a network of hydrogen bonds in this region are critical for receptor gating transitions. This network may also involve Y141, which forms hydrogen bonds with the backbone of V122, also across the interface, although the hydroxyl group of this residue appears to be less critical.²²

The hydroxyl group of Y153 is also important; its removal through the Y153F mutation caused 4–10 fold changes in EC₅₀. The structure of the 5-HT₃ receptor¹³ shows this group forming hydrogen bond interactions with other residues (Q151, R92, and Y94; Figure 4) on the complementary face. The residue equivalent to Y153 has been extensively studied in AChBP and in nACh receptors. In AChBP, many structures with bound ligands show direct or water-mediated hydrogen bonds to the backbone NH of this residue, including the varenicline-bound Ct-AChBP structure¹¹ (Figure S2B, Supporting Information), and experimental evidence for such an interaction with the backbone NH of the equivalent residue in $\alpha 4\beta 2$ nACh receptors has been demonstrated using unnatural amino acid mutagenesis.²⁵ In the $\alpha 7$ nACh receptor, the equivalent mutation shows that the EC₅₀ for varenicline increases greater than 10-fold compared to that of ACh.¹⁷ Thus, different interactions with this residue in different receptors likely contribute to drug selectivity and the observed differences in efficacy.

Possible Explanations for the Partial Agonist Behavior of Varenicline. There is substantial evidence that agonists and antagonists induce different conformational changes in the region of the C-loop with agonists causing C-loop closure around the bound ligand.^{26,27} There is also increasing evidence that partial agonists are unable to achieve the same extent of C-loop closure seen with full agonists,²⁸ although there do appear to be some exceptions.^{12,26} In the varenicline-bound structure presented here, there appears to be a small increase in the C188–W145 distance compared to that of the 5-HT bound structure. The distance between the γ -sulfur of C188 and the carbonyl oxygen of W145 was found to be 7.34 Å (an average from 5 binding sites) compared to an average distance of 6.89 Å for the 10 binding sites in the 5-HT-bound 5-HTBP structure (PDB: 2YMD), indicating that the C-loop in 5-HTBP contracts more fully over 5-HT than varenicline (although we cannot show statistical significance here due to errors associated with

the resolution and *R*-factor). A similarly increased distance has been observed for the varenicline-bound relative to the ACh-bound AChBP structure.¹¹ This intermediate level of closure of the C-loop could provide an explanation for the partial agonism achieved by this drug at the 5-HT₃ receptor. It may be, however, that the partial agonism is due to differences in interactions with the complementary face; Rohde et al.²⁹ did not observe large differences in the C188–W145 distances of a range of structures of partial agonists with varying efficacies, but did report a correlation between the strength of interactions with residues on the complementary face and the degree of efficacy. Thus, it may be that the difference between water-mediated and direct hydrogen bonds to complementary face residues with varenicline and 5-HT, respectively, results in the partial agonist activity of the former.

CONCLUSIONS

In conclusion, we show here a high resolution structure of 5-HTBP in complex with varenicline. Substitution and functional characterization of 5-HT₃ receptor residues that are equivalent to those observed in the 5-HTBP binding site suggest that the orientation of varenicline is similar in 5-HT₃ receptors, where it is a reasonably potent albeit a partial agonist. Differences in the extent of C-loop closure and/or differences in interactions with the complementary face residues could explain this partial agonist activity.

METHODS

Protein Expression and Crystallization. 5-HTBP was expressed and purified as previously described.¹⁰ A stock solution of varenicline tartrate (Sigma) was made in water at a concentration of 500 mM and mixed with 6 mg/mL 5-HTBP to yield a final varenicline concentration of 5 mM. Cryoprotection of the crystals was achieved by equilibration of the mother liquor in 5% increments of glycerol to a final concentration of 30% glycerol, and crystals were immersed in liquid nitrogen prior to X-ray diffraction data collection. The structure was determined by molecular replacement using the published structure of 5-HTBP as the template (PDB: 2YMD). After placing the ligand into the electron density map, the model of the complex was manually rebuilt and refined through iterative cycles in Coot and Phenix. The final model has an *R*_{work} value of 18.7% and *R*_{free} value of 24.4%. Model validation was carried out in Molprobit, and the model has a score of 1.75, which is in the 97th percentile for this resolution range. Figures were prepared with Pymol.

Mutagenesis. All mutant receptors were created using Quik-Change mutagenesis (Agilent). Residue numbering was altered to be consistent with mouse 5-HT₃ receptor numbering.

Cell Culture. Human embryonic kidney (HEK) 293 cells were maintained on 90 mm tissue culture plates at 37 °C and 7% CO₂ in a humidified atmosphere. They were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mix F12 (1:1) with GlutaMAX I media (Life Technologies, Paisley, UK) containing 10% HyClone fetal calf serum (GE Healthcare). For FlexStation studies, cells were transfected using polyethylenimine (PEI; Polysciences). PEI (30 μ L at 1 mg/mL), cDNA (5 μ L at 1 mg/mL; subcloned into pcDNA3.1), and DMEM (1 mL) were incubated for 10 min at room temperature, added dropwise to a 70–90% confluent plate, and incubated for 2 days. Cells were then transferred to poly-L-lysine (Cultrex)-coated 96-well plates and allowed to adhere overnight before use.

FlexStation Analysis. The methods were used essentially as described previously.³⁰ In brief, fluorescent membrane potential dye (Membrane Potential Blue kit, Molecular Devices) was diluted in flex buffer (10 mM HEPES, 115 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM glucose, pH 7.4), and 100 μ L was added to each well of transfected cells. The cells were incubated at 37 °C for 45 min, and then fluorescence was measured in a FlexStation 3 microplate

reader (Molecular Devices) at 2 s intervals for 200 s. Varenicline tartrate (Tocris) or 5-HT (Sigma) was added to each well after 20 s. The change in fluorescence (ΔF) was defined as F_{\max} (peak fluorescence) $- F_{\min}$ (baseline fluorescence). Data were normalized to the maximum ΔF with the highest concentration of 5-HT. Concentration–response data were fitted to the four-parameter logistic equation using Prism (GraphPad Software Inc., San Diego, CA).

Oocyte Maintenance and RNA Preparation. *Xenopus laevis* oocytes were purchased from Ecocyte Bioscience (Austin, TX) and stored in Barth's solution (88 mM NaCl, 2.4 mM NaHCO₃, 1 mM KCl, 0.33 mM Ca(NO₃)₂·4H₂O, 0.41 mM CaCl₂·2H₂O, 0.82 mM MgSO₄·7H₂O, and 5 mM Tris/HCl, pH 7.5) containing 2.5 mM sodium pyruvate, 50 mM gentamicin, and 0.7 mM theophylline. RNA was transcribed in vitro from Sph I-linearized plasmid cDNA template using the mMessage mMachine T7 transcription kit (Ambion, Austin, TX). Oocytes were injected with 5–20 ng of cRNA, and currents were recorded 1–2 days post injection.

Electrophysiology. Oocytes were clamped at -60 mV using a Roboocyte (Multi Channel Systems), an automated two-electrode voltage clamp workstation. Concentration–response data for each oocyte were normalized to the maximum current for that oocyte. The mean and SEM for a series of oocytes were plotted against agonist concentration and iteratively fitted to the four-parameter logistic equation using Prism. Values are presented as mean \pm SEM.

■ ASSOCIATED CONTENT

■ Supporting Information

Details of the crystallographic data, an image of the electron density of varenicline bound in 5-HTBP, and an image showing varenicline bound in AChBP. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Author Contributions

Participated in research design: S.C.R.L., K.L.P., and C.U. Conducted experiments: R.K.L., K.L.P., C.U., and S.C.R.L. Performed data analysis: R.K.L., K.L.P., C.U., S.C.R.L. Wrote or contributed to the writing of the manuscript: S.C.R.L., K.L.P., and C.U.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS:

5-HT, 5-hydroxytryptamine; nACh receptor, nicotinic acetylcholine; GABA, gamma-aminobutyric acid; HEK, human embryonic kidney; AChBP, acetylcholine binding protein

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