

# A Hydrogen Bond in Loop A Is Critical for the Binding and Function of the 5-HT<sub>3</sub> Receptor<sup>†</sup>

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**ABSTRACT:** The binding sites of Cys-loop receptors are formed from at least six loops (A–F). Here we have used mutagenesis, radioligand binding, voltage clamp electrophysiology, and homology modeling to probe the role of two residues in loop A of the 5-HT<sub>3</sub> receptor: Asn128 and Glu129. The data show that substitution of Asn128, with a range of alternative natural and unnatural amino acids, changed the EC<sub>50</sub> (from ~10-fold more potent to ~10-fold less potent than that of the wild type), increased the maximal peak current for *m*CPBG compared to 5-HT (*R*<sub>max</sub>) 2–19-fold, and decreased *n*<sub>H</sub>, indicating this residue is involved in receptor gating; we propose Asn128 faces away from the binding pocket and plays a role in facilitating transitions between conformational states. Substitutions of Glu129 resulted in functional receptors only when the residue could accept a hydrogen bond, but with both these and other substitutions, no [<sup>3</sup>H]granisetron binding could be detected, indicating a role in ligand binding. We propose that Glu129 faces into the binding pocket, where, through its ability to hydrogen bond, it plays a critical role in ligand binding. Thus, the data support a modified model of the 5-HT<sub>3</sub> receptor binding site and show that loop A plays a critical role in both the ligand binding and function of this receptor.

5-HT<sub>3</sub> receptors are members of the Cys-loop family of ligand-gated ion channels, a group that also includes nicotinic acetylcholine (nACh),<sup>1</sup> GABA<sub>A</sub>, and glycine receptors. The receptors function as a pentameric arrangement of subunits, with each subunit having a large extracellular N-terminal region and four transmembrane helices (M1–M4). The extracellular domain contains the ligand binding site, and the availability of the high-resolution structure of the acetylcholine binding protein (AChBP), which is homologous to this region, has enabled the construction of a series of homology models of the extracellular domains of several Cys-loop receptors, including nACh, GABA<sub>A</sub>, and 5-HT<sub>3</sub> receptors (1–7). These models support experimental data that indicate that ligand binding is coordinated by six noncontiguous regions, loops A–F, of the linear sequence (Figure 1A). The recent structure determination of the extracellular domain of a nACh receptor  $\alpha$  subunit provides further support for these models (8).

Studies of nACh, GABA<sub>A</sub>, and 5-HT<sub>3</sub> receptors indicate that loop A makes an important contribution to receptor function (9–13). Loop A residues Asn128, Glu129, and Phe130 are conserved in all known 5-HT<sub>3A</sub> and 5-HT<sub>3B</sub> receptor subunits (Figure 1B), and it is therefore likely that these residues are important for receptor binding and/or gating. The structure of AChBP indicates that only a single loop A residue contributes to the binding pocket, but identifying the precise 5-HT<sub>3</sub> receptor residue in the equivalent location is not straightforward, as loop A exemplifies a region in which the alignment of subunit residues with AChBP is difficult. A model of the 5-HT<sub>3</sub> receptor binding pocket predicts that the side chain of Asn128 faces into the binding pocket and interacts with 5-HT via a hydrogen bond (5), but a later study indicates that Asn128 does not participate in ligand binding (13). This study suggested a new orientation with Glu129 replacing Asn128 in the binding pocket but did not provide any experimental evidence of Glu129 mutant receptors to support this hypothesis. Phe130 has also been previously proposed as a ligand binding residue, as its substitution with Asn created receptors that respond to ACh (12), albeit at high concentrations. However, a more recent study (13) indicates that it is unlikely to be in the binding pocket, as substitutions have only small or no effects on antagonist binding, and the effect of ACh can be explained as mutations at this site can create receptors that are more sensitive to nonspecific agonists such as ACh, which will activate 5-HT<sub>3</sub> receptors at high concentrations (>1 mM). In this study, we have therefore concentrated on Asn128 and Glu129, substituting them

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<sup>1</sup> Abbreviations: Akp, 2-amino-4-ketopentanoic acid; 5-FT, 5-fluorotryptamine; 5-HT, 5-hydroxytryptamine; AChBP, acetylcholine binding protein; *m*CPBG, *m*-chlorophenylbiguanide; nACh, nicotinic acetylcholine; Nha, nitrohomalanine.

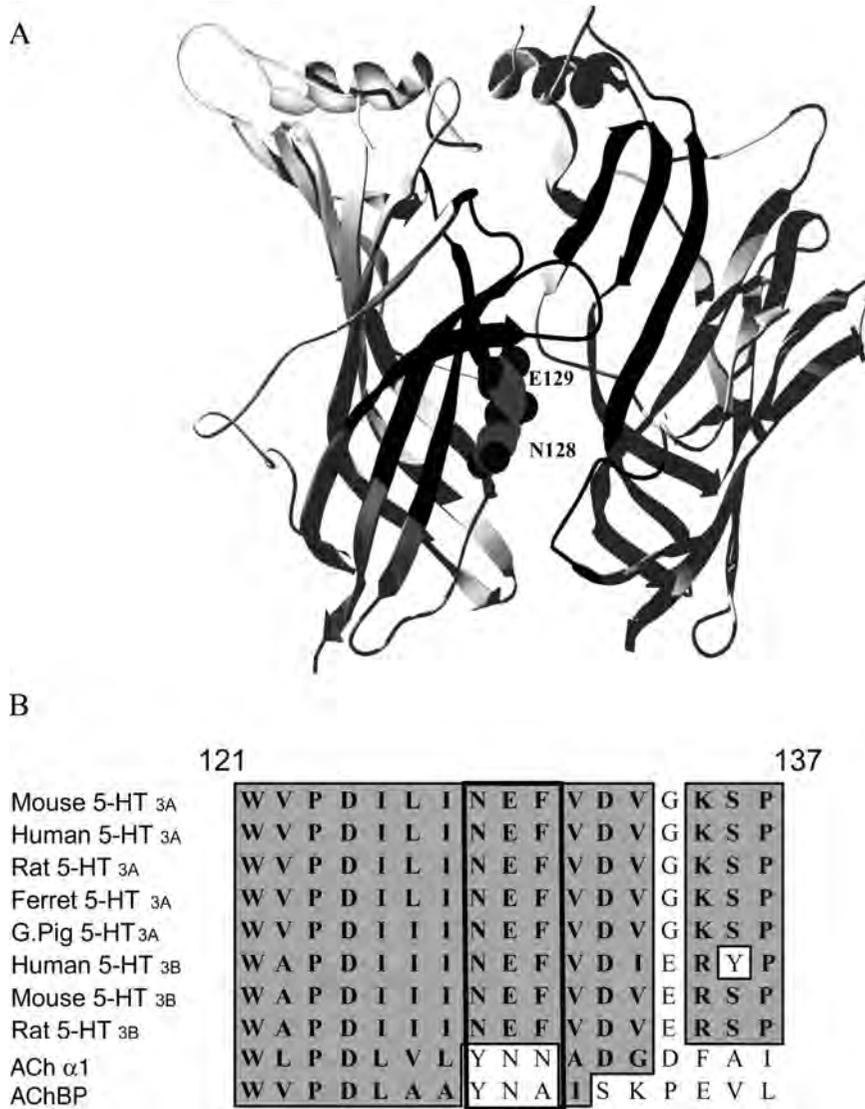


FIGURE 1: (A) Model of two adjacent subunits of the 5-HT<sub>3</sub> receptor (based on ref 5) showing the positions of the binding loops (black) and residues Asn128 and Glu129 (space filled). (B) Alignment of the binding loop A region from various 5-HT<sub>3A</sub> and 5-HT<sub>3B</sub> receptor subunits, the *Torpedo* nACh receptor α1 subunit, and AChBP. Binding loop A was originally defined as being equivalent to W121–N128 (29), but recent data suggest it may be longer (this study and ref 11). Residues with similar chemical properties are highlighted in gray. The Asn, Glu, and Phe residues conserved in all 5-HT<sub>3</sub> receptor subunits are boxed. The numbering is that of the mouse 5-HT<sub>3A</sub> receptor subunit.

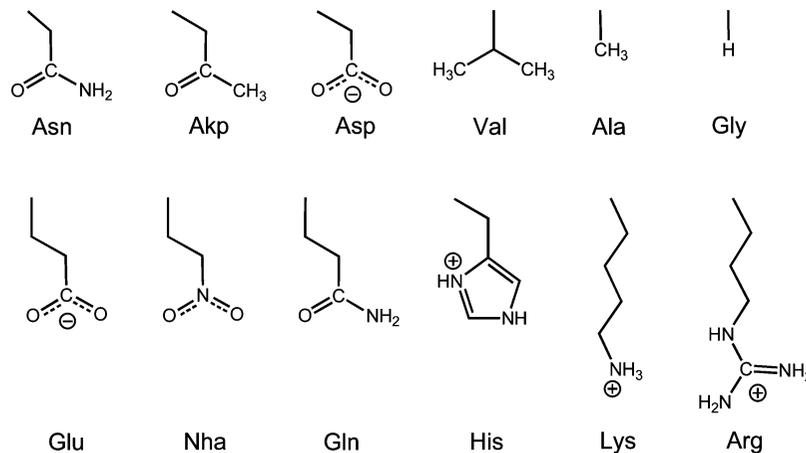


FIGURE 2: Structures of the side chains of the natural and unnatural amino acids used in these studies. Akp is aminoketopentanoic acid and Nha nitrohomoalanine.

with a range of natural and unnatural amino acids (Figure 2) to probe potential interactions with 5-HT. The data

suggest that Glu129 is directly involved in ligand binding by participating in a critical hydrogen bond with the

hydroxyl group of 5-HT, thus providing the first direct evidence that the revised model may be correct.

## EXPERIMENTAL PROCEDURES

**Mutagenesis and Preparation of cRNA and Oocytes.** Mutant 5-HT<sub>3</sub> receptor subunits were cloned into pcDNA3.1 (Invitrogen) containing the complete coding sequence for the mouse 5-HT<sub>3A</sub> receptor subunit (GenBank accession number Q6J1J7). Mutagenesis reactions were performed using the Kunkel method and confirmed by DNA sequencing. Harvested stage V–VI *Xenopus* oocytes were injected with 5 ng of cRNA produced by in vitro transcription using the mMESSAGE mMACHINE kit (Ambion) from cDNA subcloned into pGEMHE as previously described (14). The unnatural amino acids nitrohomoalanine (Nha) and 2-amino-4-ketopentanoic acid (Akp) were incorporated using nonsense suppression as previously described (14). Electrophysiological measurements were performed 24–72 h postinjection.

**Synthesis of tRNA and dCA Amino Acids.** This was conducted as described previously (14). Briefly, unnatural amino acids (Figure 2) 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<sub>CUA</sub> as detailed previously (15). Immediately prior to co-injection with cRNA, aminoacyl-tRNA was deprotected by photolysis (16). Typically, 5 ng of total cRNA was injected with 25 ng of tRNA-aa in a total volume of 50 nL. For a control, cRNA was injected with THG 74-mer tRNA (no unnatural amino acid attached).

**Characterization of Mutant Receptors.** Agonist-induced currents were recorded at 22–25 °C from individual oocytes using either conventional two-electrode voltage clamp electrophysiology or the higher-throughput automated Opus-Xpress system (MDS Axon Instruments); these two systems gave the same results. 5-HT, *m*-chlorophenylbiguanide (*m*CPBG), 5-fluorotryptamine (5-FT), and tryptamine were stored as 20–100 mM aliquots at –20 °C, diluted in Ca-free ND96 buffer [96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES (pH 7.5)]. Glass microelectrodes were backfilled with 3 M KCl and had a resistance of ~1 MΩ. The holding potential was –60 mV. To determine EC<sub>50</sub> values, concentration–response data were fitted to the four-parameter logistic equation  $I = I_{\min} + (I_{\max} - I_{\min}) / [1 + 10^{\log(\text{EC}_{50} - [A]) / n_H}]$ , where  $I_{\max}$  is the maximal response plateau,  $I_{\min}$  is the minimum response plateau,  $[A]$  is the concentration of agonist, and  $n_H$  is the Hill coefficient, using PRISM version 4.03 (GraphPad, San Diego, CA). Relative efficacies of the partial agonists *m*CPBG, 5-FT, and tryptamine are reported as  $R_{\max} = I_{\max}(\text{drug}) / I_{\max}(5\text{-HT})$ . One-way ANOVAs were performed with a Dunnett's post test to determine statistical significance. Data are quoted as means ± the standard error of the mean ( $n$ ) unless otherwise stated.

**[<sup>3</sup>H]Granisetron Binding to Oocytes.** For single-point radioligand binding assays, 20–40 oocytes were homogenized in 200 μL of 10 mM HEPES (pH 7.4) containing protease inhibitors (1 mM EDTA, 50 μg/mL soybean trypsin inhibitor, 50 μg/mL bacitracin, and 0.1 mM PMSF) and 1% Triton X-100. Following a 10 min incubation at room temperature, oocyte yolk proteins were pelleted by centrifugation at 13000g for 10 min. The supernatant was retained,

avoiding the uppermost lipid layer. Single-point assays were performed in 500 μL of 10 mM HEPES (pH 7.4) containing 25 μL of oocyte preparation and 0.5 nM [<sup>3</sup>H]granisetron (63.5 Ci/mmol; Perkin-Elmer, Inc.). Nonspecific binding was determined using 10 μM quipazine (Tocris). Tubes were incubated at 4 °C for 1 h before bound radioligand was harvested by rapid filtration onto GF/B filters presoaked in 0.3% polyethylenimine. Filters were then washed with two 3 mL washes of ice-cold HEPES buffer and left in 3 mL of scintillation fluid (Ecoscint A; National Diagnostics) for at least 4 h before scintillation counting was conducted to determine amounts of membrane-bound radioligand.

**Modeling.** The modeling was performed as described previously (13). Briefly, an alignment of the mouse 5-HT<sub>3A</sub> receptor extracellular domain (GenBank accession number Q6J1J7) with the *Lymnaea stagnalis* AChBP (GenBank accession number P58154) was performed using ClustalX and then modified by the insertion of a single-amino acid gap into the AChBP sequence following D85 (WVPD-LAAAYNAISKP) and a single-amino acid gap into the 5-HT<sub>3</sub> receptor subunit sequence following V131 (WVPDILINEFV-DVG). The new model of the 5-HT<sub>3</sub> receptor extracellular domain based on the AChBP structure (Protein Data Bank entry 1I9B) was then built using MODELER 6v2 (17) as described previously (5).

## RESULTS

Wild-type (WT) receptors displayed large, rapidly activating and desensitizing currents (Figure 3) with an EC<sub>50</sub> of 1.2 μM for 5-HT ( $p\text{EC}_{50} = 5.93 \pm 0.01$ ;  $n = 10$ ). The partial agonists *m*CPBG, 5-FT, and tryptamine had EC<sub>50</sub> values of 0.47, 18, and 120 μM, respectively (Tables 1 and 2 and Figure 4). *m*CPBG was almost as efficacious as 5-HT at these receptors, with an  $R_{\max}$  of  $0.81 \pm 0.02$  ( $n = 14$ ). The  $R_{\max}$  for 5-FT was  $0.44 \pm 0.02$  ( $n = 19$ ). However, for tryptamine, the  $R_{\max}$  was only  $0.09 \pm 0.01$  ( $n = 8$ ); these small currents precluded systematic data recording in a number of experiments.

**Asn128 Mutants.** Replacement of Asn128 with Asp, Glu, Ala, and the unnatural amino acid Akp resulted in no or small changes in 5-HT, *m*CPBG, and 5-FT EC<sub>50</sub> values, although Hill coefficients for 5-HT were reduced (Tables 1 and 2 and Figure 4). In contrast, replacement with Gln or Lys resulted in significant increases in EC<sub>50</sub> values for 5-HT, *m*CPBG, and 5-FT, while replacement with Val significantly decreased the EC<sub>50</sub>. There were no changes in Hill coefficients for these three mutants (Tables 1 and 2). For the N128R mutant, the efficacy of 5-HT appeared to be significantly reduced (Figure 5A), but responses to 5-HT were too small to allow a determination of EC<sub>50</sub>. Most of the mutations (all except Ala and Val) also resulted in changes to *m*CPBG  $R_{\max}$  values; these were increased 2–19-fold compared to that of WT (Figure 5B). There were also changes in the current profile for some mutants. N128V and N128Q substitution resulted in an apparent slower activation rate and no obvious desensitization in the continued presence of 5-HT (Figure 3B). A detailed kinetic analysis of these changes would require single-channel analyses, which are not possible with these receptors (<1 pS conductance), but the clear changes in the macroscopic data between WT and mutant receptors are consistent with changes to receptor activation and desensitization.

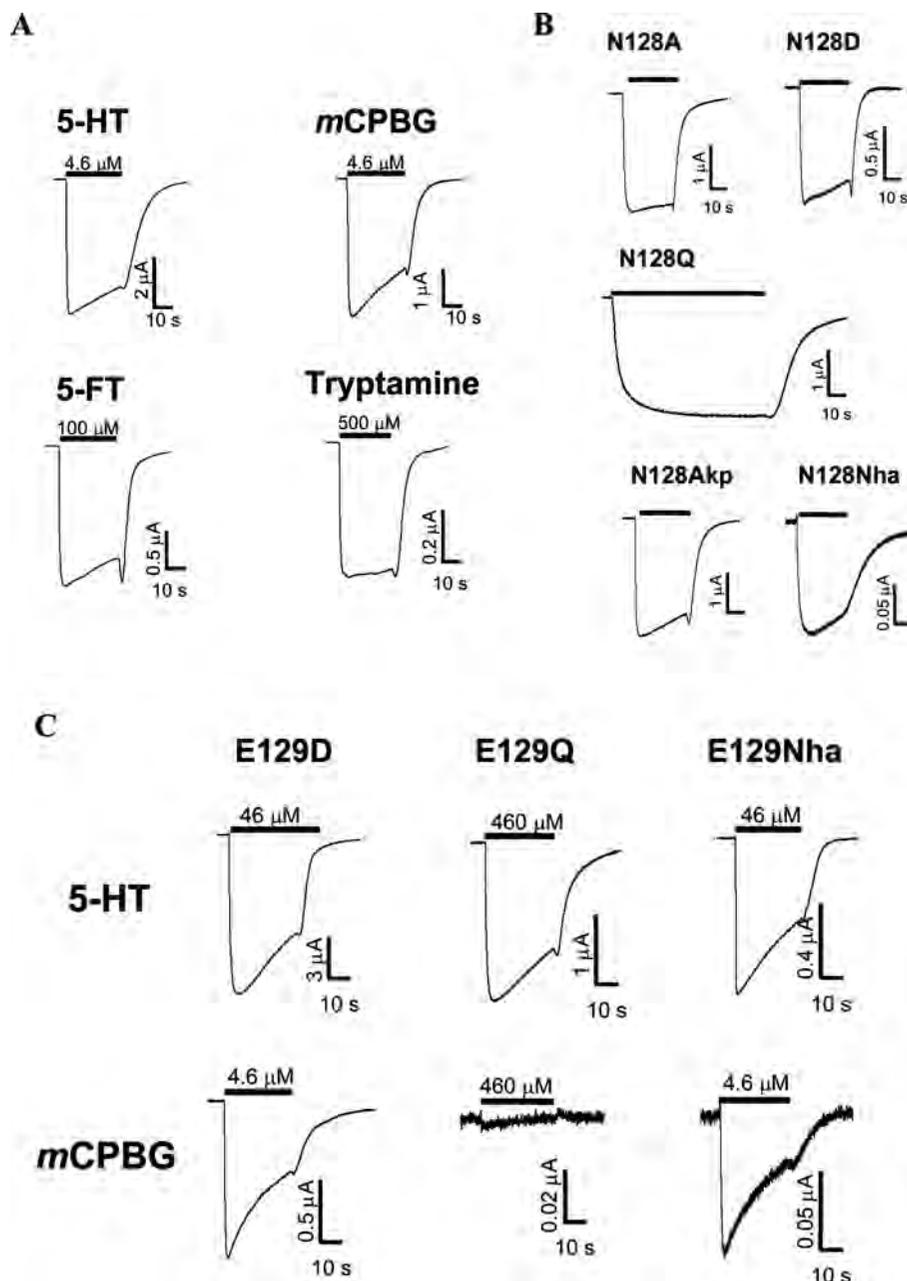


FIGURE 3: Examples of current traces. (A) Typical responses to maximal concentrations of 5-HT, *mCPBG*, 5-Ft, and tryptamine from the same oocyte expressing WT 5-HT<sub>3</sub> receptors. (B) Typical 5-HT responses of oocytes expressing Asn128 mutant receptors; [5-HT] = 22  $\mu$ M, except for N128Q (200  $\mu$ M) and N128-Nha (48  $\mu$ M). (C) Typical 5-HT and *mCPBG* responses from oocytes expressing Glu129 mutant receptors.

*Glu129 Mutants.* E129D, E129N, and E129Q exhibited robust responses to 5-HT (Figure 3C). E129H responses were small and only measurable if recorded >72 h postinjection. E129G and E129K mutants failed to respond to high concentrations (100  $\mu$ M) of either 5-HT or *mCPBG*. The unnatural amino acid Nha, which is isoelectronic and isosteric to Glu but which lacks charge, had an EC<sub>50</sub> for 5-HT similar to that of WT, as did E129D. Overall EC<sub>50</sub> values for 5-HT were in the following rank order: WT < E129D < E129Nha < E129H < E129N < E129Q (Figure 4). Hill coefficients of all the functional mutants were reduced compared to that of WT (Tables 1 and 2). Interestingly, E129Q mutant receptors failed to be activated by *mCPBG*; instead, *mCPBG* acted as an antagonist and was able to block 5-HT-induced currents, as previously reported (9). In our study, *mCPBG* blocked 100  $\mu$ M 5-HT-induced currents with an IC<sub>50</sub> of 0.63

$\mu$ M [pIC<sub>50</sub> = 6.20  $\pm$  0.04 M; *n* = 5 (Figure 6)]. Furthermore, 5-Ft, another partial agonist of 5-HT<sub>3</sub> receptors (18), also became an antagonist, blocking 100  $\mu$ M 5-HT-induced currents with an IC<sub>50</sub> of 13  $\mu$ M [pIC<sub>50</sub> = 5.26  $\pm$  0.06; *n* = 3 (Figure 6)]. Like *mCPBG*, this compound failed to activate E129Q mutant receptors on its own.

We also tested whether granisetron could inhibit 5-HT-induced responses from these mutant receptors. At E129D receptors, 10 nM granisetron was able to block 80  $\pm$  5% (*n* = 3) of the response to an EC<sub>50</sub> concentration of 5-HT and 96  $\pm$  3% at 100 nM, and recovery from granisetron block was complete in <3 min, compared with >15 min at WT receptors. Granisetron was less potent at E129N receptors, where 100 nM granisetron did not block the response to an EC<sub>50</sub> concentration of 5-HT.

Table 1: 5-HT and mCPBG EC<sub>50</sub> Values and Hill Coefficients for N128 and E129 Mutant Receptors<sup>a</sup>

receptor	5-HT pEC <sub>50</sub>	5-HT EC <sub>50</sub> (μM)	n <sub>H</sub>	mCPBG pEC <sub>50</sub>	mCPBG EC <sub>50</sub> (μM)	n <sub>H</sub>
WT	5.93 ± 0.01	1.2	2.54 ± 0.15	6.33 ± 0.02	0.47	2.03 ± 0.23
N128A	5.44 ± 0.03	3.6	1.34 ± 0.13 <sup>b</sup>	5.95 ± 0.02	1.1	1.33 ± 0.08
N128D	5.51 ± 0.01	3.1	1.63 ± 0.08 <sup>b</sup>	6.56 ± 0.03	0.27	1.54 ± 0.15
N128E	5.68 ± 0.04	2.1	1.48 ± 0.18 <sup>b</sup>	6.56 ± 0.03	0.28	1.81 ± 0.019
N128Q	4.64 ± 0.03 <sup>b</sup>	23	2.11 ± 0.29	5.52 ± 0.18 <sup>b</sup>	3.0	2.41 ± 0.16
N128R	SR	SR	SR	5.14 ± 0.02 <sup>b</sup>	7.3	1.93 ± 0.16
N128K	4.47 ± 0.03 <sup>b</sup>	34	2.13 ± 0.38	5.41 ± 0.03 <sup>b</sup>	3.9	1.43 ± 0.16
N128V	7.04 ± 0.02 <sup>b</sup>	0.091	3.18 ± 0.60	7.13 ± 0.02 <sup>b</sup>	0.074	5.07 ± 0.85 <sup>b</sup>
N128-Akp	5.33 ± 0.01 <sup>b</sup>	4.6	1.49 ± 0.06 <sup>b</sup>	ND	ND	ND
N128-Nha	SR	SR	SR	5.55 ± 0.02 <sup>b</sup>	2.8	2.18 ± 0.23
E129A	NR	NR	NR	NR	NR	NR
E129D	5.73 ± 0.03	1.9	1.81 ± 0.16 <sup>b</sup>	6.60 ± 0.10	0.25	1.19 ± 0.31
E129G	NR	NR	NR	NR	NR	NR
E129H	4.85 ± 0.09 <sup>b</sup>	14	1.07 ± 0.24 <sup>b</sup>	6.43 ± 0.04	0.37	1.63 ± 0.20
E129K	NR	NR	NR	NR	NR	NR
E129N	4.25 ± 0.02 <sup>b</sup>	56	1.17 ± 0.07 <sup>b</sup>	6.21 ± 0.05	0.62	1.25 ± 0.19
E129Q	3.93 ± 0.01 <sup>b</sup>	120	1.55 ± 0.07 <sup>b</sup>	NR	NR	NR
E129-Nha	5.45 ± 0.04	3.5	1.18 ± 0.12 <sup>b</sup>	6.25 ± 0.07	0.56	1.91 ± 0.60

<sup>a</sup> Data are means ± the standard error of the mean ( $n = 3-16$ ). NR indicates no response. SR indicates small (<100 nA) responses. ND indicates not determined. <sup>b</sup> Significant difference  $p < 0.05$  and for pEC<sub>50</sub> values >3-fold different from that of WT.

Table 2: 5-HT and Tryptamine EC<sub>50</sub> Values and Hill Coefficients for N128 and E129 Mutant Receptors<sup>a</sup>

receptor	5-HT pEC <sub>50</sub>	EC <sub>50</sub> (μM)	n <sub>H</sub>	tryptamine pEC <sub>50</sub>	EC <sub>50</sub> (μM)	n <sub>H</sub>
WT	4.75 ± 0.02	18	2.71 ± 0.24	3.93 ± 0.01	120	2.86 ± 0.14
N128A	5.00 ± 0.06	10	2.34 ± 0.75	4.04 ± 0.03	91	2.90 ± 0.68
N128D	4.75 ± 0.02	18	2.61 ± 0.28	SR	—	—
N128E	4.86 ± 0.02	14	1.97 ± 0.18	4.23 ± 0.03	59	2.90 ± 0.53
N128Q	4.03 ± 0.05 <sup>b</sup>	94	2.38 ± 0.58	3.62 ± 0.02	240	3.10 ± 0.39
E129D	5.08 ± 0.04	8.3	1.95 ± 0.30	SR	—	—
E129N	4.93 ± 0.04	12	1.25 ± 0.13 <sup>b</sup>	SR	—	—

<sup>a</sup> Data are means ± the standard error of the mean ( $n = 3-13$ ). SR indicates small (<100 nA) responses. <sup>b</sup> Significant difference  $p < 0.05$  and for EC<sub>50</sub> values >3-fold different from that of WT.

**Binding Data.** We have previously examined both Asn128 and Glu129 mutant receptors expressed in HEK cells (13). For Asn128 mutant receptors, there were no significant differences in [<sup>3</sup>H]granisetron binding affinity for any substitution studied, while no specific binding was observed for any Glu129 mutant receptor, at concentrations up to 20 nM. In the study presented here, we examined single-point [<sup>3</sup>H]granisetron binding to solubilized oocyte preparations (it requires very large numbers of oocytes to create [<sup>3</sup>H]granisetron saturation binding curves and therefore is not practical). No specific radioligand binding was observed at 0.5 nM [<sup>3</sup>H]granisetron for E129A, E129G, and E129K mutant receptors, while levels of binding in Asn128 receptors were similar to those in WT receptors (Figure 7). These data suggest that Glu129 substitutions ablate high-affinity antagonist binding, but at least some substitutions permit agonist binding, as large (>5 μA) responses to 5-HT and mCPBG were observed for E129D and E129N receptors.

## DISCUSSION

The data described here support a modified 5-HT<sub>3</sub> receptor homology model (13), in which Glu129, rather than Asn128, faces into the binding pocket. The data indicate a critical hydrogen bond between Glu129 and the hydroxyl of 5-HT, which places this residue firmly in the binding pocket. Asn128 may play a role in receptor gating, but the data show that it is not directly involved in binding ligands, as previously proposed (5).

Loop A was identified many years ago as a region that contributes to ligand binding in nACh receptors; affinity

labeling with [<sup>3</sup>H]ACh mustard indicated the positive charge of ACh was positioned near the loop A residue Tyr93 (19). There was also evidence for a contribution from neighboring Asn94 (20), and a detailed functional analysis of Asp97 has led to the proposal that loop A could be compared to a latch, which holds the channel closed in the absence of agonists, and reduces the probability of channel opening (11). More recent studies, in particular the high-resolution structure determination of AChBP, confirm the importance of the loop A Tyr at position 89 (equivalent to Tyr93 in nAChR) which is in close contact with bound ligands (21). The aligned Tyr is also important in GABA<sub>A</sub> receptors; Tyr97 in the β2 subunit has recently been shown to make a cation-π interaction with GABA (22). It was therefore not surprising that the aligning residue in the 5-HT<sub>3</sub> receptor, Asn128, was considered to be important. Homology modeling identified it as the only loop A residue in the binding pocket and predicted a hydrogen bond between Asn128 and 5-HT (5). However, experimental studies have cast some doubt on this conclusion, as changing Asn128 did not affect [<sup>3</sup>H]granisetron binding affinity (13).

Our new data, incorporating both natural and unnatural amino acids at this position, provide a detailed analysis of the role of Asn128 and strongly suggest that Asn128 has its most significant role in the conformational change that results in receptor gating. All Asn128 mutant receptors exhibited changes in their functional characteristics (Figure 2), but these were particularly evident in N128Q receptors. Gln has chemical properties similar to those of Asn, yet this mutation markedly slows apparent current activation, increases the

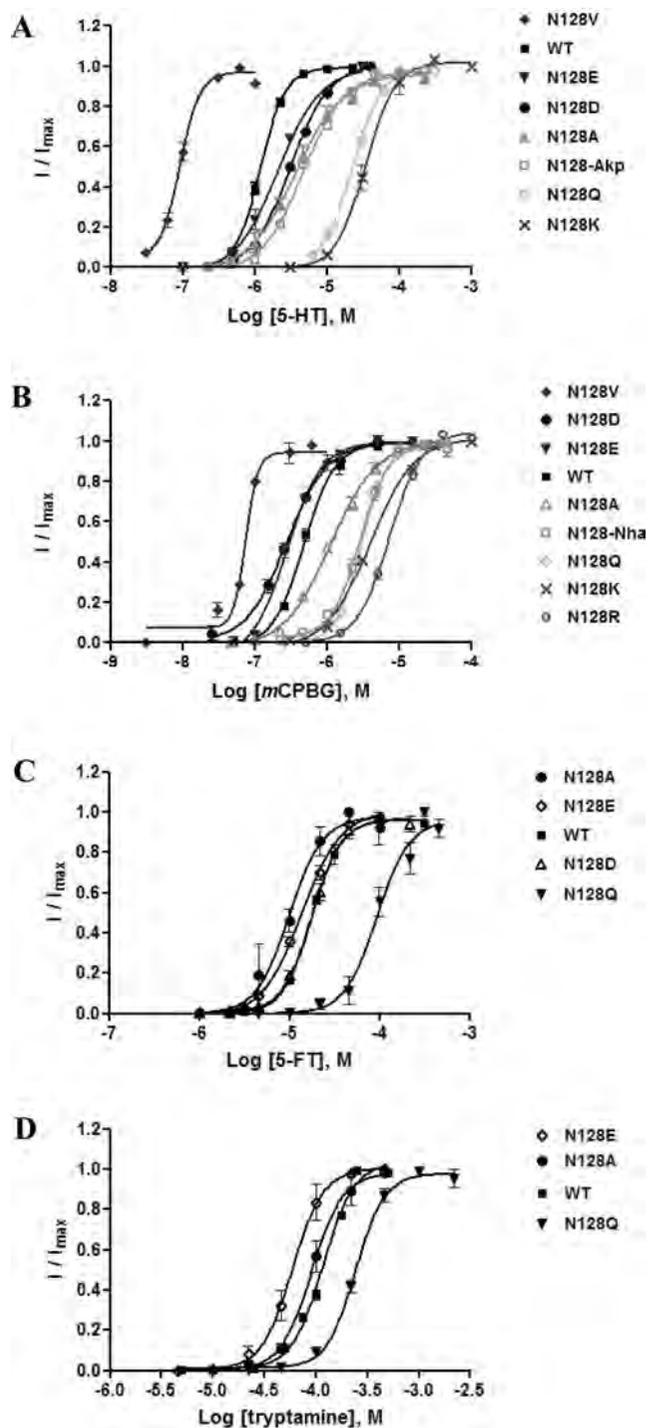


FIGURE 4: Concentration–response data for wild-type (WT) and mutant 5-HT<sub>3</sub> receptors. Data are means  $\pm$  the standard error of the mean. Parameters of the fitted curves are listed in Tables 1 and 2.

relative efficacy ( $R_{max}$ ) of the partial agonist *mCPBG*, and eliminates receptor desensitization (Figures 3 and 4). Changes in current activation and  $R_{max}$  strongly suggest effects on receptor opening, and while desensitization is not well understood, it is known to be influenced by channel opening and closing rates and the rates of conformational changes to and from the desensitized state. These observations therefore all suggest that Asn128 has a role in facilitating transitions between conformational states rather than direct effects upon ligand binding. In the new model, this residue is close to loop B, especially Thr179, and both these residues contribute

to a complex network of hydrogen bonds that could potentially be involved in the conformational change that results in receptor gating.

Receptors with substitutions at Glu129 have, in the past, been insufficiently characterized due to problems with low levels of expression (9, 13). In this study, these problems have been largely overcome by the use of *Xenopus* oocytes as expression hosts. Large responses to 5-HT and the partial agonists *mCPBG* and 5-Ft were measured with mutants of Glu129 that did not previously display measurable currents when expressed in HEK293 cells. Interestingly, only the Glu129 mutant receptors in which Glu was replaced with residues that have the ability to accept a hydrogen bond responded robustly to 5-HT application, suggesting that this property is critical for 5-HT binding. Previously published ligand docking data have indicated that the hydroxyl of 5-HT is located in this region of the binding pocket, and in the new model, this hydroxyl would donate a hydrogen bond to Glu129; more specifically, one of the side chain O atoms of Glu129 would interact with the hydrogen of the 5-HT 5-hydroxyl (Figure 8). Note that an ionic interaction involving Glu129 is not supported by our data with the unnatural amino acid *Nha*. This amino acid is structurally similar to Glu: The nitro group is planar, like the carboxylate, and the two N–O bonds are of equal length, as are the C–O bonds in the carboxylate. Two resonance structures are possible (as with carboxylate), but in a nitro group, the N atom carries a positive charge and the O atoms share a negative charge; thus, overall the group is neutral, in contrast to the negative charge on a carboxylate. A nitro group could therefore not contribute to an ionic bond. As there was no significant increase in  $EC_{50}$  when *Nha* was substituted for Glu, it shows that an ionic bond is not formed here. *Nha* could, however, still form a hydrogen bond as each O in the nitro group has two lone pairs of electrons (as does the carboxylate), which can serve as hydrogen bond acceptors.

Interestingly, mutations at Glu129 have no effect on the  $EC_{50}$  values of the partial agonists *mCPBG* and 5-Ft. This might be expected with *mCPBG*, which has a structure distinct from that of 5-HT and is unlikely to interact with identical binding site residues, but the only difference between 5-HT and 5-Ft is the group at the 5 position. The OH group of 5-HT is a good hydrogen bond donor and a moderately good hydrogen bond acceptor; however, the F of 5-Ft cannot donate a hydrogen bond and is a very poor hydrogen bond acceptor. Thus, if 5-Ft binds in the same orientation as 5-HT, which seems likely, it is probable that there is no hydrogen bond here with Glu129, a hypothesis that is supported by the data. The lack of this bond may be the reason why 5-Ft acts as only a partial agonist.

If Glu129 interacts directly with 5-HT, then it must face into the binding site and could interact with antagonists. Our, and previously published, data support this proposal: there is no specific [<sup>3</sup>H]granisetron binding to Glu129 mutant receptors in either HEK cells or oocyte membranes in the usual subnanomolar range (13). Interestingly, though, granisetron does appear to be able to bind to E129D mutant receptors at higher concentrations, as 10 nM granisetron inhibited  $\sim$ 80% of 5-HT-induced currents [WT  $IC_{50}$  = 0.2 nM (23)]. Combined with the fact that E129D mutant receptors recover more quickly than WT receptors from granisetron inhibition, these data suggest that E129D mutant

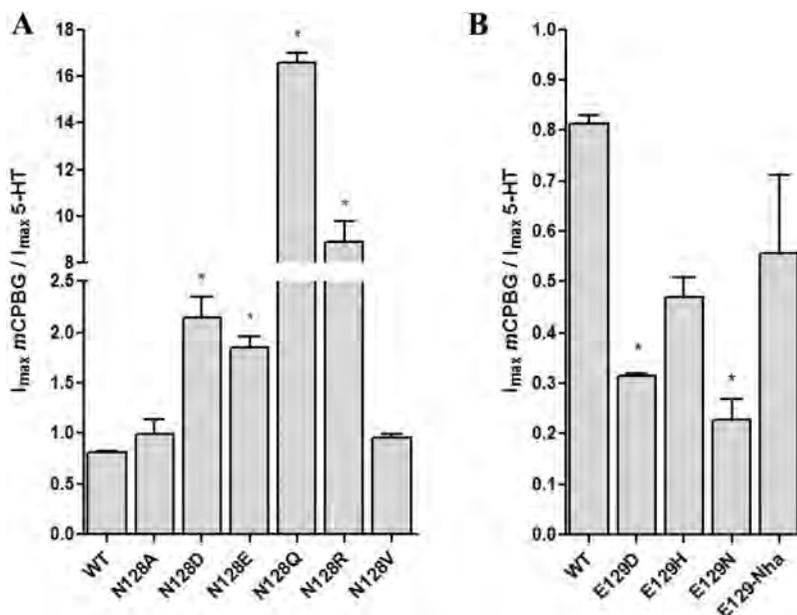


FIGURE 5: Relative efficacy ( $R_{max}$ ) of *mCPBG* at WT and (A) N128 and (B) E129 mutant receptors. Data are means  $\pm$  the standard error of the mean ( $n > 3$ ). Note the substantial change in the range of efficacies in panel B vs panel A. The asterisk indicates a value significantly different from that of WT ( $p < 0.05$ ).

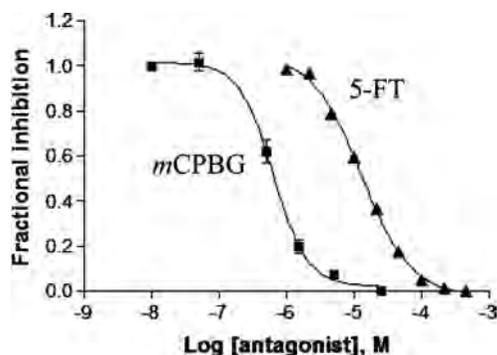


FIGURE 6: *mCPBG* and 5-FT are antagonists at E129Q receptors. Concentration–response data showing inhibition of the 100  $\mu$ M 5-HT-induced response. Each agonist was co-applied with 5-HT. Responses are normalized to the response to 5-HT alone. Data are means  $\pm$  the standard error of the mean ( $n = 3-6$ ).

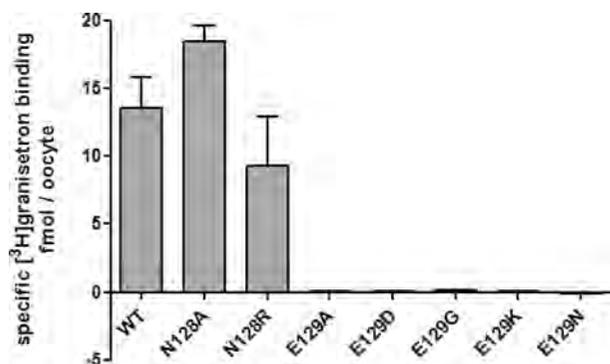


FIGURE 7: Antagonist binding to 5-HT<sub>3</sub> receptors expressed in oocytes. Specific binding of 0.5 nM [<sup>3</sup>H]granisetron to oocyte membrane samples. Data are means  $\pm$  the standard error of the mean ( $n = 4$ ).

receptors have a faster dissociation rate constant for granisetron. Such an explanation is consistent with previous equilibrium radioligand binding studies, where an  $\sim$ 100-fold increase in the granisetron  $K_d$  was reported (9).

Our data also reveal small but significant changes in relative efficacies for *mCPBG* at functional Glu129 mutant

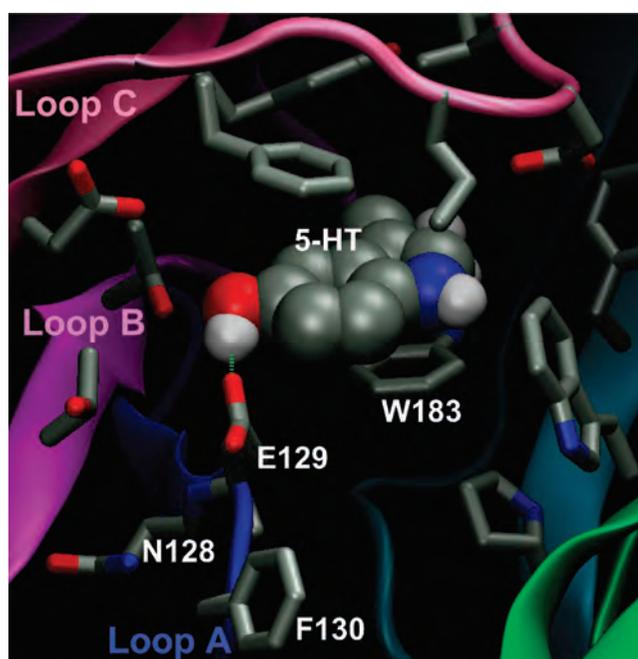


FIGURE 8: New model of 5-HT<sub>3</sub> receptor binding site, showing 5-HT hydrogen bonded to Glu129. This model is that described by Sullivan et al. (13) in which a single-amino acid gap was inserted into the 5-HT<sub>3A</sub> receptor subunit sequence (GenBank accession number Q6J1J7) following V131 (WVPDILINEFV-DVG). The new model of the complete mouse 5-HT<sub>3A</sub> receptor extracellular domain was then built using *L. stagnalis* AChBP (GenBank accession number P58154, PDB entry 1I9B) as a template. The locations of Asn128, Glu129, and Trp183 relative to 5-HT are shown. The proposed H-bond between Glu129 and the hydroxyl group of 5-HT is colored green.

receptors, indicating there also may be a role for this residue in the conformational changes leading to receptor gating. These changes are opposite to those we observed with Asn128. We do not yet understand what this implies, although it may be related to the different roles of the two residues and/or distinct mechanisms of action or critical binding residues used by different agonists. In support of

this latter hypothesis, a similar study on a series of loop C residues, which are also proposed to play roles in binding and/or gating, revealed increases in *mCPBG* efficacy but decreases in the efficacy of another partial agonist, 2-methyl-5-HT, in the same mutant receptors (24). In our study, the conversion of *mCPBG* from a partial agonist to an antagonist at E129Q mutant receptors could reflect a change in the affinity of *mCPBG* for certain conformational states of the receptor only (e.g., a reduction in affinity of the open state but not the closed state). This would correspond to the “K” phenotype of allosteric receptor mutants described by Galzi et al. (25).

The importance of Glu129 suggests it may be equivalent to Tyr93 in the nACh receptor, which has also been proposed to play a role in both binding and function. Mutating Tyr93 results in a rightward shift of the dose–response curve (26), mainly because of slower ligand association and channel opening rate constants (27). Similarly, the equivalent residue in the GABA<sub>A</sub> receptor,  $\beta_2$ Tyr97, which directly contacts GABA through a cation– $\pi$  interaction (22), may also be involved in gating; mutation to Cys causes spontaneous activation (10). Aligning Glu129 and Tyr93 requires that a space be inserted in the conserved WxPDxxxN domain in loop A of the nACh receptor. This sequence is critical for locating the B loop in the nACh receptor through interactions involving Asp89 (28). More recent data, however, show that in non-ACh receptors the xxxN portion of this region may not be critical; in the GABA<sub>A</sub> receptor, for example, two amino acid “spaces” must be inserted in the ‘xxx’ tract to allow  $\beta_2$ Tyr97 to contribute to the binding pocket. We therefore propose that Glu129 is equivalent to Tyr93 and faces into the binding pocket, where it forms a hydrogen bond with the 5-OH group of 5-HT.

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