Transducing Agonist Binding to Channel Gating Involves Different Interactions in 5-HT₃ and GABAₐ Receptors*

Kerry L. Price*, Katherine S. Millen*, and Sarah C. R. Lumniss†‡§

From the †Department of Biochemistry, University of Cambridge and §Neurobiology Division, MRC-LMB, Hills Rd., Cambridge CB2 2QH, United Kingdom

5-Hydroxytryptamine (5-HT₃) and γ-aminobutyric acid, type C (GABAₐ) receptors are members of the Cys-loop superfamily of neurotransmitter receptors, which also includes nicotinic acetylcholine, GABAₐ, and glycine receptors. The details of how agonist binding to these receptors results in channel opening is not fully understood but is known to involve charged residues at the extracellular/transmembrane interface. Here we have examined the roles of such residues in 5-HT₃ and GABAₐ receptors. Charge reversal experiments combined with data from activation by the partial agonist β-alanine show that in GABAₐ receptors there is a salt bridge between Glu-92 (in loop 2) and Arg-258 (in the pre-M1 region), which is involved in receptor gating. The equivalent residues in the 5-HT₃ receptor are important for receptor expression, but charge reversal experiments do not restore function, indicating that there is not a salt bridge here. There is, however, an interaction between Glu-215 (loop 9) and Arg-246 (pre-M1) in the 5-HT₃ receptor, although the coupling energy determined from mutant cycle analysis is lower than might be expected for a salt bridge. Overall the data show that charged residues at the extracellular/transmembrane domain interfaces in 5-HT₃ and GABAₐ receptors are important and that specific, but not equivalent, molecular interactions between them are involved in the gating process. Thus, we propose that the molecular details of interactions in the transduction pathway between the binding site and the pore can differ between different Cys-loop receptors.

There have been many biochemical and functional studies on different members of the Cys-loop family of ligand-gated ion channels, but the prototypic member of this family, the neuromuscular nicotinic acetylcholine (nACh) receptor, is still the best understood. This is the only receptor for which reasonably high resolution structural information (4 Å) is available from cryo-electron microscopy studies (1), and further knowledge has come from x-ray crystal structures of acetylcholine binding proteins (AChBP), which are homologous to the extracellular domain of this receptor (2, 3). Cys-loop receptors are composed of five pseudo-symmetrically arranged subunits surrounding a central ion-conducting pore. Each subunit is composed of an extracellular, a transmembrane, and an intracellular domain. The extracellular domain (ECD) contains the ligand binding site, which is formed at the interface of two adjacent subunits by the convergence of three amino acid loops (A–C) from one (the principal) subunit and three β-strands (D–F) from the adjacent (or complementary) subunit. The transmembrane domain (TMD) contains four membrane spanning α-helices (M1–M4) and a short C terminus. M2 from each subunit lines the pore and contains regions responsible for channel gating and ion selectivity. A large loop between M3–M4 forms the intracellular domain and is involved in channel conductance and modulation.

Structural details extrapolated from AChBP have greatly enhanced our understanding of the extracellular, ligand binding, N-terminal domain. However, we have considerably less knowledge of the molecular details of the transmembrane region and know little about the mechanism by which information from agonist binding results in the opening of a pore some 30–60 Å distant. It is becoming increasingly clear that some of the machinery involved in coupling ligand binding to channel gating is located at the extracellular/transmembrane interface and involves loops 2, 7, and 9 in the ECD, the pre-M1 region, and the M2–M3 loop in the transmembrane domain. Specific evidence to support this includes classic mutagenesis experiments of these regions, the creation of an AChBP-5-HT₃ receptor chimera (comprised of AChBP and the TMD of the 5-HT₃ receptor), which only functioned when loops 2, 7, and 9 were replaced with their 5-HT₃ receptor equivalents, and a study using unnatural amino acids which showed that indeed a proline located at the apex of the M2–M3 loop in the 5-HT₃ receptor can control pore opening via a cis-trans isomerization (4–8).

These data have indicated that the interaction between the ECD and TMD is responsible for the functional coupling of ligand binding to channel opening, but there is controversy as to whether this is a hydrophobic interaction (1), pairwise ionic interaction (5, 6), or more global ionic attractions (8). One might expect a mechanism of channel opening to be conserved within the Cys-loop superfamily; however, the hydrophobic residue (nACh α Val-46) proposed by Unwin (1) to be involved in gating is not conserved in all Cys-loop receptors, nor are the charged residues in the GABAₐ receptor M2–M3 and β₁–β₂
loops studied by Kash et al. (5). Some charged residues, however, are conserved, including the cluster of basic residues in the pre-M1 region, and studies have shown the importance of these residues in GABA_A, 5-HT_3, and nACh receptors (6, 9). Here we have explored the role of conserved charged residues in two members of the Cys-loop family, the 5-HT_3 receptor and the GABA_C receptor, which is a member of the GABA_A receptor family, and in particular explored if there is a salt bridge between this region and the B1-B2 loop as has been demonstrated in the nACh receptor (6). The data suggest that the specific interactions between the ECD and TMD can vary between different Cys-loop receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture reagents were obtained from Invitrogen, except fetal calf serum, which was from Labtech International. GABA_A receptor antisera was from Santa Cruz Biotechnology, Inc. Biotinylated anti-rabbit IgG, fluorescein isothiocyanate avidin D, and Vectashield mounting medium were from Vector. [3H]Granisetron (63.5 Ci/mmol) was from PerkinElmer Life Sciences, and quipazine dimaleate was from Tocris. All other reagents were of the highest obtainable grade.

**Site-directed Mutagenesis**—Mutant mouse 5-HT_3A receptor subunits (GenBank accession number value) or human GABA_C (rho1) receptor subunits (GenBank accession number M62400, kindly gifted by D. S. Weiss) were prepared in the vector pcDNA3 using standard techniques (10). Mutant sequences were subcloned into the vector pGEMHE (11) for expression in Xenopus oocytes.

**Oocyte Preparation**—This was as described previously (12). Briefly, harvested stage V-VI Xenopus oocytes were washed in 4 changes of OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2, 5 mM HEPES, pH 7.5), de-folliculated in 1.5 mg/ml collagenase for ~1 h, washed again in 4 changes of OR2, and transferred to 70% Leibovitz media (Invitrogen) buffered with 10 mM HEPES, pH 7.5. The following day they were injected with 5 ng of mRNA produced by in vitro transcription using the mMESSAGE mACHINE kit (Ambion) from wild type or mutant DNA. Electrophysiological measurements were performed 24–72 h post-injection.

**Electrophysiological Recordings**—Two-electrode voltage clamping of Xenopus oocytes was performed using standard electrophysiological procedures as previously described (12) with minor modifications. Briefly, a GeneClamp 500B amplifier was connected to a PC running CLAMPED Version 6.0.3 software via a DigiData1200 Series Interface (all Axon Instruments, Inc.). Glass microelectrodes were pulled from GC150TF-10 glass capillaries (Harvard Apparatus) using a P-87 micropipette puller (Sutter) to a resistance of 0.5–1 megaohms and backfilled with 3 M KCl. Oocytes were maintained at a holding potential of ~60 mV unless stated otherwise and perfused continuously with ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2, 5 mM HEPES, pH 7.5) for GABA_A receptor experiments or calcium-free ND96 for 5-HT_3 receptor experiments at a rate of 3–4 ml/min. GABA or 5-HT was applied to the bath using a Valve Bank 8 II system (Automate Scientific, Inc.). Concentration-response curves and parameters were obtained using Prism v4 software (GraphPad).

**Cell Culture**—Human embryonic kidney 293 cells were maintained on 90-mm tissue culture plates at 37 °C and 7% CO_2 in a humidified atmosphere. They were cultured in Dulbecco’s modified Eagle’s medium/nutrient mix F12 (1:1) with GLUTAMAX™ containing 10% fetal calf serum and passaged when confluent. Cells were transfected using calcium phosphate precipitation (13) at 80–90% confluency. After transfection, cells were incubated for 3–4 days before assay.

**Immunofluorescent Localization**—The methodology was as described for localization of the 5-HT_3 receptor (12). Briefly, transfected cells, grown on 22-mm coverslips, were fixed in ice-cold 4% paraformaldehyde and then incubated with the GABA_C receptor antiserum sc-21336, which recognizes the extracellular domain. Biotinylated anti-goat IgG and fluorescein isothiocyanate avidin D were used to detect bound antibody as per the manufacturer’s instructions. Coverslips were mounted in Vectashield mounting medium, and immunofluorescence was observed using a Nikon optiphot or confocal microscope.

**[^3H]Granisetron Oocyte Binding**—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, 0.1 mM phenylmethylsulfonyl fluoride) and 1% Triton X-100. After a 10-min incubation at room temperature, oocyte yolk proteins were pelleted by centrifugation at 13,000 × g 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 [^3H]granisetron. Non-specific binding was determined using 10 μM quipazine. Tubes were incubated at 4 °C for 1 h before bound radioligand was harvested by rapid filtration onto GF/B filters pre-soaked in 0.3% polyethyleneimine. Filters were then washed with two 3-ml washes of ice-cold HEPES buffer and radioactivity determined by scintillation counting.

**Double Mutant Cycle Analysis**—EC_{50} values were used to calculate the coupling coefficient (Ω) based on the equation Ω = EC_{50}^{wt,mut,mut}/EC_{50}^{wt,wt}. EC_{50}^{wt,mut,mut} and EC_{50}^{mut,wt} represent the wild type (WT) receptor, wt,mut and mut,wt represent single mutations, and mut,mut represents the double mutant incorporating both single mutations. The coupling energy between these two residues was then calculated using ΔG_{int} = −RTlnΩ.

**RESULTS**

**5-HT_3 Receptor Characterization**

Application of 5-HT to Xenopus oocytes expressing WT 5-HT_3 receptors produced concentration-dependent, rapidly activating inward currents that desensitized over the time-course of the application. Concentration response curves revealed an EC_{50} of 1.2 μM and Hill slope of 2.5 ± 0.2. Cys-loop receptor subunit alignments allowed us to identify charged residues (Asp, Glu, Lys, or Arg) belonging to 5-HT_3 receptor loops 2, 7, 9, and the pre-M1 region (Fig. 1). Unlike other members of the Cys-loop LGIC superfamily, the 5-HT_3 receptor M2-M3 loop and post-M4 regions do not have any charged residues. On a homology model of the 5-HT_3 receptor, those residues close to the ECD-TMD interface that could
interact with other charged residues (Glu-80, Asp-172, Glu-213, Arg-244, Arg-245, and Arg-246) were mutated to have a similar, opposite, or no charge.

**Single Mutations**—Mutations to the charged interface residues were not well tolerated; no functional response could be detected for 7 of 15 mutations (Table 1). Some of these (Arg 245 and some Asp-172 and Glu-80 mutants) were probably due to lack of receptor expression; no \[^3H\]granisetron binding could be detected. In addition, some mutant receptors had responses that were too small to obtain concentration-response curves (E80A, E80C, and E213R).

Those mutant receptors that did respond to 5-HT (e.g. Table 1 and Fig. 2) mostly had similar parameters to WT receptors (K81Q, K81R, E215R, and R244E). The exception was R246E mutant receptors, which had an EC\(_{50}\) of 0.08 \(\mu\)M.

**Multiple Mutations**—The lack of response in the E80R and R245E mutants could not be rescued by the double mutation. Most other charge reversal double, triple, and quadruple mutations also were nonfunctional (Table 2). Swapping charges in the combination E215R/R246E, however, produced a functional receptor (Fig. 2) which had an EC\(_{50}\) for 5-HT of 0.35 \(\mu\)M that is between the EC\(_{50}\)s for receptors displaying the single mutations E215R (1.13 \(\mu\)M) and R246E (0.076 \(\mu\)M). This finding that the impairment of channel function seen with R246E can be partially recovered by adding the E215R mutation suggests that these two residues interact. Double mutant cycle analysis...
of this pairing supports this suggestion and indicates an interaction energy of $-0.9$ kcal mol$^{-1}$ (Fig. 2). This is lower than values previously seen for a buried salt bridge (6, 14), but it does suggest a specific interaction indicating these residues are physically close (15).

**TABLE 1**

Characteristics of mutant 5-HT$_3$ receptors

| Receptor | 5-HT pEC$_{50} \pm$ S.E. | 5-HT EC$_{50}$ | Hill coefficient | Binding |
|----------|--------------------------|---------------|------------------|---------|
| WT       | 5.93 ± 0.011             | 1.2           | 2.54 ± 0.15      | +++     |
| E80A     | SR                       |               |                  |         |
| E80C     | SR                       |               |                  |         |
| R245Q    | NR                       |               |                  |         |
| R245E    | NR                       |               |                  |         |
| D172R    | SR                       |               |                  |         |
| E213R    | NS                       |               |                  |         |
| E215R    | 5.95 ± 0.021             | 1.13          | 2.24 ± 0.27      |         |
| D172A    | NR                       |               |                  |         |
| D172E    | 6.52 ± 0.044             | 0.30          | 2.17 ± 0.56      |         |
| D172N    | NR                       |               |                  |         |
| D172R    | NR                       |               |                  |         |
| R244E    | 6.24 ± 0.015             | 0.58          | 2.81 ± 0.27      | +       |
| R245D    | NR                       |               |                  |         |
| R245E    | NR                       |               |                  |         |
| R245Q    | NR                       |               |                  |         |
| R246E    | 7.12 ± 0.011             | 0.076         | 3.08 ± 0.20      |         |

* Significantly different from WT, $p < 0.05$.

**GABA$_C$ Receptor Characterization**

Oocytes injected with WT GABA$_C$ receptor mRNA displayed robust, non-desensitizing inward currents in response to GABA application (Fig. 3). Concentration-response curves revealed an EC$_{50}$ of 1.08 $\mu$m and a Hill coefficient of 1.8 ± 0.2 (Table 3).

**Mutations in the Pre-M1 Region—**Oocytes injected with R258A, R258E, or R258K GABA$_C$ receptor subunit mRNA did not respond to GABA application up to a concentration of 30 mM; oocytes injected simultaneously with WT GABA$_C$ receptor subunit mRNA responded to GABA, indicating that receptor expression was unaffected. In contrast, oocytes expressing Arg-257 mutant receptors produced large inward currents in response to GABA, with EC$_{50}$ values and Hill coefficients not significantly different from wild type (Table 3). Immunofluorescent studies with antisera that recognizes the extracellular domain of the GABA$_C$ receptor revealed that R258A but not R258E or R258K receptors were expressed at the cell surface (Fig. 3).

**Mutation at Glu-92—**The responses from oocytes expressing E92A and E92D mutant receptors had EC$_{50}$ values ~5- and 10-fold lower than WT receptors, respectively (Table 4). E92D receptors also had a reduction in their Hill coefficients. The shapes of the responses indicated slower off rates for all Glu-92 mutants, and determination of $\tau_{off}$ revealed ~3-fold increases compared with WT receptors (Table 5).

To further explore the role of this residue, we examined the effect of the partial agonist $\beta$-alanine, which has an $R_{max}$ ($I_{max}$ GABA) of 0.49 ± 0.02 ($n = 4$) in wild type receptors. In E92A and E92D mutant receptors $\beta$-alanine was found to act as a full agonist (Fig. 4). E92R mutant receptors did not respond to high concentrations of GABA (up to 30 mM) or $\beta$-alanine (up to 100 mM) but were expressed at the cell surface (Fig. 3).

**Multiple Mutations—**The lack of response in the E92R and R258E mutants was rescued by the double mutation E92R/R258E. This receptor has an EC$_{50}$ of 0.48 $\mu$m, ~2-fold lower than that for wild type receptors (1.08 $\mu$m) but not as low as the values we observed for E92A or E92D receptors (0.22 and 0.10 $\mu$m, respectively). The kinetics of the response in the double

**TABLE 2**

Characteristics of double and triple mutant 5-HT$_3$ receptors

| Receptor | 5-HT pEC$_{50} \pm$ S.E. | 5-HT EC$_{50}$ | Hill coefficient | $n$ | Binding |
|----------|--------------------------|---------------|------------------|----|---------|
| WT       | 5.93 ± 0.011             | 1.2           | 2.54 ± 0.15      | 10 | +++     |
| E80R/R244E | NR                       |               |                  |    |         |
| E80R/R246E | NR                       |               |                  |    |         |
| E80R/R245E | NR                       |               |                  |    |         |
| D172R/R245D | NR                       |               |                  |    |         |
| D172R/R246D | NR                       |               |                  |    |         |
| E213R/R244E | NR                       |               |                  |    |         |
| E213R/R245E | NR                       |               |                  |    |         |
| E213R/R245E | SR                       |               |                  |    |         |
| E215R/R244E | 6.01 ± 0.005             | 0.98          | 3.35 ± 0.15      | 3  |        |
| E215R/R245E | NR                       |               |                  |    |         |
| E215R/R246E | 6.46 ± 0.017             | 0.35          | 3.03 ± 0.35      | 4  |        |
| E80R/R245E/R246D | NR                       |               |                  |    |         |
| D172R/R245E/R246D | NR                       |               |                  |    |         |
| E80R/D172R/R245E/R246D | NR               |               |                  |    |         |

* Significantly different from WT, $p < 0.05$. 

**FIGURE 2.** Mutant cycle analysis of WT, E215R, R246E, and E215R+R246E receptors. For each receptor, typical responses to maximal concentrations of 5-HT are shown and the free energy changes for each part of the cycle are indicated.
mutant also revealed an increased $\tau_{\text{off}}$ (Table 5), and $\beta$-alanine again acted as a full (96 ± 3%) rather than a partial agonist, as we had observed for the E92A and E92D receptors (Fig. 4). The lack of response of the E92R and R258E mutant receptors unfortunately precluded double mutant cycle analysis of this pairing.

**DISCUSSION**

Understanding the method of transduction between the ligand binding site and the pore is a key goal in the Cys-loop receptor field. It is known that charged residues located at the interface of the extracellular and transmembrane domains are important, and salt bridges between such residues have been demonstrated in both nACh and GABA$_A$ receptors, although the molecular details of these are different. Here we have explored the possibility of a salt bridge between a conserved Glu residue in loop 2 and a conserved Arg residue in the pre-M1 subunit residues Glu-92 and Arg-258 are highly conserved in all Cys-loop receptors; they are equivalent to Glu-45 and Arg-209 in the nACh receptor, it is part of the transduction pathway. Further evidence to support this hypothesis comes from our studies with the partial agonist $\beta$-alanine, which acted as a full agonist in the double mutant receptors. A change in the efficacy of a partial agonist is typically interpreted as a change in gating.
5-HT<sub>3</sub> and GABA<sub>C</sub> Receptors Have Distinct Gating Interactions

Parameters, as it indicates that the mutation has affected the open probability (<i>P</i><sub>open</sub>) of the receptor; here, the small <i>P</i><sub>open</sub> in the wild type receptor has increased in the mutant receptor. These data combined with the recent work of Wang et al. (16), which indicates that the salt bridge at in the GABA<sub>C</sub> receptor is required to stabilize the closed state and is broken when the receptor moves into the open state, provide good evidence that a E92R/R258E salt bridge is involved in receptor gating and, thus, support our hypothesis that this salt bridge is a critical part of the transduction pathway coupling agonist binding to channel gating in the GABA<sub>C</sub> receptor.

Interestingly, responses obtained from the double mutant receptors did have some differences to wild type receptors; they had a slower off rate, and the EC<sub>50</sub> was ~2-fold lower than wild type GABA responses (Tables 2 and 5). This does not detract from our proposal that a salt bridge here is intimately involved in receptor function but indicates that the structure of the region may be subtly altered by the mutations. In contrast, nACh receptor E45R/R209E double mutants were reported to restore function to that of wild type (6), suggesting both that a salt bridge between these two residues is crucial for receptor function and that the mutations cause no other structural or functional changes.

The single mutations were also informative. No Arg-258 mutations were functional, and some were also not expressed at the membrane. Interestingly a similar effect has been observed for some other Cys-loop receptors mutated at the equivalent position: α<sub>1</sub>K220Cβ<sub>2</sub> GABA<sub>A</sub> receptors, for example, showed no specific [<sup>3</sup>H]muscimol binding, whereas α<sub>1</sub>β<sub>2</sub>R216C receptors bound similar amounts to wild type receptors, although they did not respond to GABA (17). Similarly the glycine receptor mutation α<sub>1</sub>R218Q, which has been found in some patients suffering from hyperekplexia, has dramatic effects on receptor expression (~10% of wild type levels) and function (~200-fold decrease in agonist sensitivity (18)), and Torpedo nACh receptor R209E mutant receptors also show little or no expression at the cell surface (6).

Substitution of Glu-92 with Ala or Asp did result in functional receptors. Their macroscopic kinetics, however, were different from wild type receptors, with slowed off rates (Fig. 3 and Table 5) and decreased EC<sub>50</sub>s (Table 4). In these functional Glu-92 mutants, β-alanine again acted as a full agonist, inferring that receptor gating is affected by these mutations. In the nACh receptor E45K and E45D mutant receptors also have decreased EC<sub>50</sub>s compared with wild type, and E45Q and E45R mutants have altered partial agonist efficacy, again indicating an effect on gating (8). In addition, single channel measurements with E45K mutant receptors have confirmed an effect on gating with shortened channel openings and lengthened channel closings (6).

We should also mention that in the GABA<sub>C</sub> receptor there are two adjacent Arg residues in the pre-M1 region; mutation of Arg-257, however, had no significant effect on GABA-induced responses, indicating that it is not involved in receptor function. Previous studies also support this interpretation; cysteine mutations of the residues equivalent to Arg-257 in the rat GABA<sub>A</sub> receptor α<sub>1</sub> and β<sub>2</sub> subunits (Arg-119 and Lys-215, respectively) yield receptors with similar GABA EC<sub>50</sub> values as wild type receptors (17), and even reversing the charge in R209E mutant receptors also show little or no expression at the cell surface (6).

The 5-HT<sub>3</sub> Receptor Interface Differs from That of GABA<sub>C</sub> Receptors—Our new data support previous studies (20, 21) which show that charged residues at the ECD/TMD interface are important for the function of the 5-HT<sub>3</sub> receptor. There are, however, significant differences between our 5-HT<sub>3</sub> receptor data and those we obtained for the GABA<sub>C</sub> receptor; in particular there appears to be no salt bridge between Glu-80 and Arg-258 (the residues equivalent to GABA<sub>C</sub> receptor Glu-92 and Arg-258).

Glu-80 was sensitive to reverse charge mutation; E80R was nonfunctional, although small responses were observed with E80C and E80A mutants. E80R appears to prevent receptor expression; no [<sup>3</sup>H]granisetron binding could be detected in oocytes expressing these receptors. Thus, the role of Glu-80 in the 5-HT<sub>3</sub> receptor differs from that in nACh receptors where...
the charge reversal mutants in this position yielded functional receptors with increased sensitivity to agonist (8).

Substitution of Arg-245 with Glu also appears to prevent receptor expression as does the equivalent substitution in the nACh receptor (6); R245E mutant receptors were nonfunctional, and no [3H]granisetron binding could be detected. Previous work has shown that no response to 5-HT could be detected with R245A mutant receptors (20), although no experiments to check receptor expression were performed. The same authors also suggested that Arg-246 (Arg-222 by their numbering) forms an important link between binding and gating (21), and interestingly, their mutant R246A receptors had similar changes in properties to the GABA_α-Glu-92 mutants, with the partial agonist 2-Me-5-HT becoming a full agonist and the 5-HT EC-50 5-fold lower than in wild type receptors. We observed that both R244E and R246E were functional, and R246E had an EC-50 15-fold less than wild type. These data combined with the Hu et al. data (21) suggest that Arg-246 in the 5-HT_3 receptor may have an analogous role to Glu-92 and Glu-45 in GABA_α and nACh receptors, respectively.

Because Glu-80 does not appear to form a salt bridge with Arg-245, we explored other possible partners. Asp-172 was particularly interesting as the equivalent residue has been shown to be important for function in glycine, nACh, and GABA_α receptors; indeed, in GABA_α receptors it is proposed to form a salt bridge with the M2-M3 loop residue Lys-279 (5). It is highly conserved and, thus, as expected, sensitive to reverse charge mutation; D172R was nonfunctional. A double reverse charge mutant (D172E/R245D), however, was also nonfunctional, indicating a salt bridge here is unlikely. However, D172E receptors had an EC-50 close to wild type receptors, and D172N receptors, although nonfunctional, did reach the cell surface, unlike D172A and D172R receptors. This suggests that a negatively charged residue is important for expression in this receptor and also appears to have a role in function. The residue does not, however, appear to form a salt bridge with Arg-245.

Glut-213 and Glu-215 are also possible partners for Arg-245, but multiple reverse charge mutants with these residues indicated no salt bridges. E215R, however, could rescue R246E mutation, and these residues are also physically close on a model of the 5-HT_3 receptor (Fig. 5). Double mutant cycle analysis suggested there is an interaction here, although the relatively small difference in coupling energy (0.9 kcal/mol) suggested this may not be a salt bridge; the nACh receptor Glu45/Arg-209 salt bridge had a coupling energy of 3.1 kcal/mol. Thus, our data to date would support the proposal that charge residues are important at the ECD/TMD interface but indicate that there is no specific salt bridge that is critical for receptor function.

In conclusion, the data strongly indicate that there is a salt bridge between Glu-92 and Arg-258 in the GABA_α receptor, as previously demonstrated for the equivalent residues in the nACh receptor. The situation appears quite distinct from that at the 5-HT_3 receptor ECD/TMD interface, where there is no evidence for a salt bridge between the equivalent residues, although there does appear to be an interaction between a different pair of residues, Glu-215 and Arg-246. Thus, assuming that the mechanism of channel opening is similar in all these receptors, our data support the hypothesis that it is the global charge pattern at the ECD/TMD interface that is important for transducing ligand binding into channel gating and not specific molecular interactions between equivalent residues. We await evidence from high resolution structural data to test this hypothesis.

REFERENCES

1. Unwin, N. (2005) J. Mol. Biol. 346, 967–989
2. Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van Der Oost, J., Smit, A. B., and Sixma, T. K. (2001) Nature 411, 269–276
3. Celie, P. H., Klaassen, R. V., van Rossum-Fikkert, S. E., van Elk, R., van Nierop, P., Smit, A. B., and Sixma, T. K. (2005) J. Biol. Chem. 280, 26457–26466
4. Bouzat, C., Gumilar, F., Spitzmaul, G., Wang, H. L., Rayes, D., Hansen, S. B., Taylor, P., and Sine, S. M. (2004) Nature 430, 896–900
5. Kash, T. L., Jenkins, A., Kelley, J. C., Trudell, J. R., and Harrison, N. L. (2003) Nature 421, 272–275
6. Lee, W. Y., and Sine, S. M. (2005) Nature 438, 243–247
7. Lummis, S. C., Beene, D. L., Lee, L. W., Lester, H. A., Broadhurst, R. W., and Dougherty, D. A. (2005) Nature 438, 248–252
8. Xiu, X., Hanek, A. P., Wang, J., Lester, H. A., and Dougherty, D. A. (2005) J. Biol. Chem. 280, 41655–41666
9. Vicente-Aguillo, F., Rovira, J. C., Sala, S., Sala, F., Rodríguez-Ferrer, C., Campos-Caro, A., Criado, M., and Ballesta, J. J. (2001) Biochemistry 40, 8300–8306
10. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
5-HT₃ and GABAᵥ Receptors Have Distinct Gating Interactions

11. Liman, E. R., Tytgat, J., and Hess, P. (1992) Neuron 9, 861–871
12. Price, K. L., and Lummis, S. C. (2004) J. Biol. Chem. 279, 23294–23301
13. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
14. Vaughan, C. K., Harryson, P., Buckle, A. M., and Fersht, A. R. (2002) Acta Crystallogr. D Biol. Crystallogr. 58, 591–600
15. Schreiber, G., and Fersht, A. R. (1995) J. Mol. Biol. 248, 478–486
16. Wang, J., Lester, H. A., and Dougherty, D. A. (July 2, 2007) J. Biol. Chem., DOI 10.1074/jbc.M702314200
17. Mercado, J., and Czajkowski, C. (2006) J. Neurosci. 26, 2031–2040
18. Castaldo, P., Stefanoni, P., Miceli, F., Coppola, G., Del Giudice, E. M., Bellini, G., Pascotto, A., Trudell, J. R., Harrison, N. L., Annunziato, L., and Taglialetela, M. (2004) J. Biol. Chem. 279, 25598–25604
19. Kash, T. L., Dizon, M. J., Trudell, J. R., and Harrison, N. L. (2004) J. Biol. Chem. 279, 4887–4893
20. Zhang, L., Hosoi, M., Fukuzawa, M., Sun, H., Rawlings, R. R., and Weight, F. F. (2002) J. Biol. Chem. 277, 46256–46264
21. Hu, X. Q., Zhang, L., Stewart, R. R., and Weight, F. F. (2003) J. Biol. Chem. 278, 46583–46589