0026-895X/11/7904-742–748$20.00
MOLECULAR PHARMACOLOGY
Vol. 79, No. 4
69583/3678856
Mol Pharmacol 79:742–748, 2011
Printed in U.S.A.

A Cation-π Interaction at a Phenylalanine Residue in the Glycine Receptor Binding Site Is Conserved for Different Agonists

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Received October 22, 2010; accepted January 4, 2011

ABSTRACT

Cation-π interactions have been demonstrated to play a major role in agonist-binding in Cys-loop receptors. However, neither the aromatic amino acid contributing to this interaction nor its location is conserved among Cys-loop receptors. Likewise, it is not clear how many different agonists of a given receptor form a cation-π interaction or, if they do, whether it is with the same aromatic amino acid as the major physiological agonist. We demonstrated previously that Phe159 in the glycine receptor (GlyR) α1 subunit forms a strong cation-π interaction with the principal agonist, glycine. In the current study, we investigated whether the lower efficacy agonists of the human GlyR β-alanine and taurine also form cation-π interactions with Phe159. By incorporating a series of unnatural amino acids, we found cation-π interactions between Phe159 and the amino groups of β-alanine and taurine. The strengths of these interactions were significantly weaker than for glycine. Modeling studies suggest that β-alanine and taurine are orientated subtly differently in the binding pocket, with their amino groups further from Phe159 than that of glycine. These data therefore show that similar agonists can have similar but not identical orientations and interactions in the binding pocket and provide a possible explanation for the lower potencies of β-alanine and taurine.

Introduction

Glycine receptors (GlyRs) are ligand-gated chloride channels expressed mainly at inhibitory synapses in brain stem and spinal cord (Lynch, 2004). In these tissues, GlyRs contribute to generation of motor rhythm, coordination of reflex circuits, and processing of sensory signals (Legendre, 2001; Harvey et al., 2004). Along with $GABA_A$ receptors, 5-hydroxytryptamine$_3$ (5-HT_3) receptors, and nicotinic acetylcholine receptors (nAChRs), GlyRs belong to the Cys-loop receptor family (Le Novère and Changeux, 2001). All Cys-loop members are composed of five subunits that are pseudosymmetrically arranged around a central ion-conducting pore. Individual subunits consist of a large N-terminal ligand-binding domain and four transmembrane helices that are connected by loops of varying size (Unwin, 1995; Miyazawa et al., 2003). Structural studies on acetylcholine-binding protein (AChBP) (Brejc et al., 2001) and prokaryotic Cys-loop receptor homologs (Hilf and Dutzler, 2008; Bocquet et al., 2009; Hilf and Dutzler, 2009) have confirmed the location of the agonist binding site at the interface of adjacent subunits. Three loops from one subunit (A–C) and three β strands from the adjacent subunit (D–F) create the binding pocket. Like all Cys-loop receptors, the GlyR binding site is lined with aromatic residues, and we have shown previously that a cation-π interaction between the positively charged amine of glycine and Phe159 in loop B makes a substantial contribution to agonist binding (Pless et al., 2008). Likewise, other studies have shown that aromatic side chains form cation-π interactions with the principal agonists of other Cys-loop receptors, such as nACh, 5-HT_3, MOD-1 (modulation of locomotion defective 1), and $GABA_A$ receptors (Zhong et al., 1998; Beene et al., 2002; Mu et al., 2003; Lummis et al., 2005;

This work was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grants NS11756, NS34407]; the Wellcome Trust [Grant RG81925]; a Wellcome Trust Senior Research Fellowship in Basic Biomedical Science (to S.C.R.L.); the European Union (Grant FP7NeuroCypres); the Australian Research Council; and the National Health and Medical Research Council of Australia.

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Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.110.069583.

ABBREVIATIONS: GlyR, glycine receptor; 5-HT_3, 5-hydroxytryptamine$_3$; nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine-binding protein; tRNA, transfer RNA; WT, wild type.

Padgett et al., 2007; Dougherty, 2008). However, the nature and location of the aromatic residue that interacts with the cation is not conserved among Cys-loop receptors or among different agonists for a given receptor (Beene et al., 2002; Mu et al., 2003; Dougherty, 2008; Xiu et al., 2009). Recent kinetic studies on the mechanism of partial agonism (Lape et al., 2008) have sparked a renewed interest in the question how partial agonists differ in their mechanism of action. Although a number of studies have established how glycine binds to the GlyR (Grudzinska et al., 2005; Pless et al., 2008), little is known about the binding mode of other GlyR agonists, such as β-alanine and taurine, which are both endogenous amino acid agonists of the GlyR (Schmieden et al., 1992; Lewis et al., 2003). β-Alanine and taurine are structurally similar to glycine (Fig. 1A), and all agonists compete for the same binding site (Schmieden et al., 1992; Schmieden and Betz, 1995) According to a single-channel kinetic analysis, dissociation rates for β-alanine and taurine are increased 2.5- and 4-fold, respectively (compared with glycine), and efficacy by 2- and 5-fold, respectively (Lewis et al., 2003). To explore the molecular mechanism(s) involved in these differences, we used the nonsense suppression method (Dougherty, 2000; Beene et al., 2003) to incorporate unnatural amino acids into the GlyR binding site to determine whether β-alanine and taurine, like glycine, form a cation-π interaction with a phenylalanine residue at position 159.

Materials and Methods

Mutagenesis and Preparation of mRNA and Oocytes. For optimal expression in oocytes, the human GlyR α_1 subunit cDNA was subcloned into the pGEMHE vector. The QuikChange mutagenesis kit (Stratagene, La Jolla, CA) was used for site-directed mutagenesis. Successful incorporation of mutations was confirmed by automated sequencing. Capped mRNA for oocyte injection was generated using the mMessage mMachine kit (Ambion, Austin, TX). *Xenopus laevis* (Nasco, Fort Atkinson, WI) oocytes were prepared as described previously (Pless et al., 2007) and injected with mRNA, either alone or with tRNA. After injection, oocytes were incubated for 18 to 36 h at 18°C.

Synthesis of tRNA and dCA Amino Acids. The procedure used here has been described previously (Beene et al., 2004). In short, unnatural amino acids (as shown in Fig. 1) were chemically synthesized as nitroveratryloxycarbonyl-protected cyanomethyl esters and coupled to the dinucleotide dCA. The resulting product was subsequently ligated enzymatically to a 74-mer THG73 $tRNA_{CUA}$ as described previously (Nowak et al., 1998). The aminoacyl tRNA was deprotected by photolysis (Kearney et al., 1996) directly before coinjection with the mRNA. Typically, 10 to 15 ng of mRNA were coinjected with 20 to 30 ng of tRNA-amino acid in a total volume of 50 nl. In control experiements, mRNA was injected alone or together with tRNA-dCA (with no amino acid attached). Under these conditions, we never observed measurable currents, even at high (5 mM) concentrations of agonist ($n = 6$). This result confirmed that the constructs containing the TAG stop codon generated truncated and hence nonfunctional receptors. It also suggests that no endogenous amino acids from the oocyte are incorporated at these sites and, finally, that the tRNAs are not reacylated with naturally occurring amino acids by endogenous synthetases.

Characterization of Mutant Receptors. The OpusXpress voltage-clamp system (Molecular Devices, Union City, CA) was used to record peak currents from individual oocytes induced by agonist. All recordings were performed at 22–25°C. Glycine, β-alanine, and taurine (Sigma, St. Louis, MO) were stored at −20°C as 1 M and 500 mM aliquots, respectively. They were diluted in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4) directly before the experiments. Delivery to the cells was achieved using the computer-controlled perfusion system of the OpusXpress. Glass microelectrodes had resistances between 0.5 and 3 MΩ and were backfilled with 3 M KCl. All recordings were performed at a holding potential of −60 mV. The empirical Hill equation, fitted with a nonlinear least-squares algorithm, was used to obtain half-maximal concentrations (EC_{50}) and Hill coefficient (n_H) values for ligand-induced activation (SigmaPlot 9.0; Systat Software, Point Richmond, CA). Data are presented as mean ± S.E.M.

Modeling. This was performed as described previously (Reeves et al., 2003; Thompson et al., 2005). Three-dimensional models of the extracellular region of the glycine receptor were built using MODELLER 6, version 2 (http://salilab.org/modeller/) based on the crystal structure of acetylcholine-binding protein in the agonist-bound state (carbamylcholine; Protein Data Bank ID 1UV6). The model was energy-minimized in SYBYL version 6.8 (Tripos Inc., St. Louis, MO) using the AMBER force field by moving side chains alone (Weiner et al., 1984). Models and predicted hydrogen bonds were viewed using ViewerLite version 5.0 (Accelrys, San Diego, CA). Docking was performed using GOLD 3.0 (The Cambridge Crystallographic Data Centre, Cambridge, UK) as described previously (Thompson et al., 2007) with the ligands constrained to dock within 4.5 Å of Arg65, because a previous study has convincingly demonstrated that Arg65 is a common anchor point for the carboxyl termini of different agonists (e.g., glycine and taurine) (Grudzinska et al., 2005).

Results

Locating the Residues of Interest Using Conventional Mutagenesis. In this study, we chose to focus on

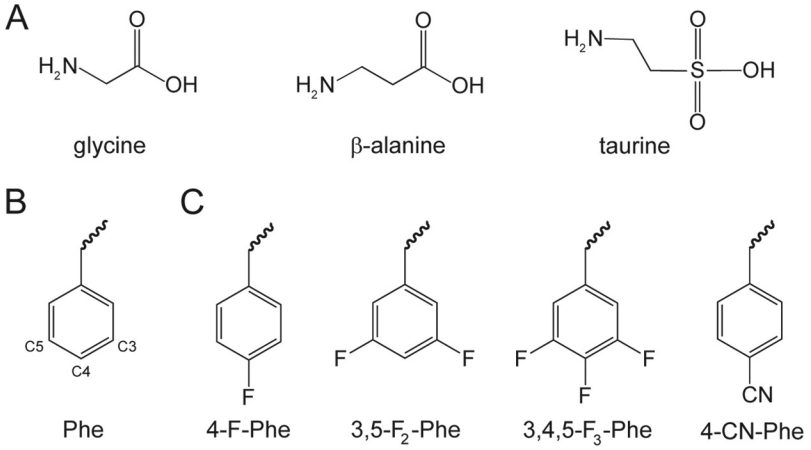

Fig. 1. Structures of the agonists used in this study (A), as well as Phe (B) and unnatural Phe analogs (C) incorporated at positions 63, 159, and 207.

Phe63, Phe159, and Phe207. Mutations at these sites markedly affect receptor function, and aligning residues in other receptors formed cation-π interactions in previous studies (Rajendra et al., 1995b; Grudzinska et al., 2005; Pless et al., 2008). F63A substitution caused increases in EC_{50} values of 760-, 280-, and 360-fold for glycine, β-alanine, and taurine, whereas F63Y substitution had smaller effects on glycine and β-alanine responses (17- and 57-fold increases in EC_{50}, respectively), but no responses were detected with taurine, perhaps indicating that size here is critical for taurine binding/activation (Table 1).

F159Y substitution decreased EC_{50} values for all three agonists as reported previously (Schmieden et al., 1993), whereas F159A substitution caused large increases (70- – 260-fold) (Table 1). F207A substitution eliminated all agonist responses. F207Y had no significant effect on EC_{50} values. This mutation did, however, decrease R_{max} (Table 1). Other aromatic residues near the GlyR binding pocket, such as Phe99, Phe100, and Tyr202 could also potentially contribute to ligand binding, but previous studies using conventional mutagenesis have ruled out cation-π interactions at these sites (see *Discussion* for details).

Characterization of the Phe63TAG Construct. Incorporating a Phe at position 63 with the nonsense suppression method resulted in functional receptors with EC_{50} values similar to those of wild-type (WT) receptors (De Saint Jan et al., 2001; Pless et al., 2008). We thus conclude that the WT phenotype was successfully "rescued" by coinjection of Phe63TAG mRNA and Phe tRNA. A single fluorination (4-F-Phe) at position 63 resulted in a small decrease in EC_{50} for β-alanine or taurine, respectively (Tables 2 and 3). In contrast, introduction of Phe derivatives with two or three fluorines (3,4-F_2-Phe and 3,4,5-F_3-Phe) resulted in 10- and 5-fold increases in the β-alanine EC_{50}, respectively (compared with WT). Because the EC_{50} values for 4-F-Phe are lower than those for Phe for both β-alanine and taurine, and there is no pattern in the EC_{50} values for 3,4-F_2-Phe and 3,4,5-F_3-Phe with β-alanine, we conclude that neither agonist forms a cation-π interaction with Phe63.

Phe159 Forms a Cation-π Interaction with β-Alanine. Successive addition of fluorine atoms to Phe derivatives at position 159 results in a stepwise increase in the β-alanine EC_{50} (Fig. 2, A, C, and E; Table 2). These results strongly suggest a cation-π interaction between β-alanine and Phe159. The data include not only fluorinated analogs, but also a Phe derivative with a cyano group (CN) in the 4 position. 4-CN-Phe is slightly larger in size than the fluorinated Phe derivatives but is well tolerated in Cys-loop receptor binding sites (Padgett et al., 2007; Pless et al., 2008). This substitution yielded a β-alanine EC_{50} between those for 3,4-F_2-Phe and 3,4,5-F_3-Phe, consistent with the cation-π binding ability of 4-CN-Phe.

A plot of the data reveal a strong linear correlation between the relative log EC_{50} value (scaled to WT) and the cation-π binding ability of the incorporated Phe derivative (Fig. 3A), consistent with a cation-π interaction between β-alanine and Phe159 (Mecozzi et al., 1996; Zhong et al., 1998). The slope of this linear fit was significantly lower than that of glycine (Fig. 3C).

Comparing glycine-induced currents (Fig. 2, B and D) with β-alanine-induced currents (Fig. 2, A and C) reveals that 3,4,5-F_3-Phe, but not Phe, markedly reduces receptor desensitization for β-alanine-induced currents. This may suggest that a strong cation-π binding ability at position 159 is necessary for WT-like ligand-binding and gating properties.

Phe159 Also Forms a Cation-π Interaction with Taurine. Introduction of Phe derivatives with a range of cation-π interaction energies revealed that Phe159 also forms such an

TABLE 1

Dose-response data for GlyRs with conventional mutations at positions 63, 159, and 207

Data are presented as mean ± S.E.M. (n = 3–8).

	Glycine	β-Alanine	Taurine
WT EC_{50}	100 ± 13	303 ± 61	522 ± 75
R_{max}		0.9 ± 0.03	0.9 ± 0.04
F63A EC_{50}	89,000 ± 6000	115,000 ± 8,000	>500,000
R_{max}		0.4 ± 0.05	
F63Y EC_{50}	2,000 ± 400	23,000 ± 3,000	N.R.
R_{max}		1.0 ± 0.08	
F159A EC_{50}	8,100 ± 200	104,000 ± 17,000	126,000 ± 32,000
R_{max}		1.1 ± 0.05	0.9 ± 0.07
F159Y EC_{50}	13.2 ± 1.0	9.3 ± 0.7	23.3 ± 4
R_{max}		1.4 ± 0.04	1.0 ± 0.11
F207A EC_{50}	N.R.	N.R.	N.R.
R_{max}			
F207Y EC_{50}	161 ± 23	520 ± 88	754 ± 122
R_{max}		0.6 ± 0.06	0.7 ± 0.04

R_{max}, ratio of maximal currents induced by β-alanine or taurine versus glycine; N.R., no response at 100 mM.

TABLE 2

β-Alanine dose-response data for GlyRs with unnatural amino acids incorporated at positions 63, 159, and 207

Data are presented as mean ± S.E.M.

	EC_{50}	n_H	$R_{(\beta\text{-Ala/Gly})}$	n
	μM			
63-Phe (WT)	315 ± 16	1.6 ± 0.1	1.0 ± 0.3	6
63-F-Phe	123 ± 4*	2.2 ± 0.1	1.1 ± 0.4	5
63-F_2-Phe	3890 ± 150*	1.6 ± 0.1	0.5 ± 0.2	4
63-F_3-Phe	1590 ± 210*	1.6 ± 0.4	0.2 ± 0.1	4
159-Phe (WT)	373 ± 8	2.0 ± 0.1	0.8 ± 0.1	4
159-F-Phe	470 ± 33*	1.9 ± 0.2	0.9 ± 0.3	5
159-F_2-Phe	3290 ± 120*	1.7 ± 0.1	0.7 ± 0.2	6
159-CN-Phe	11,500 ± 500*	1.6 ± 0.1	0.7 ± 0.1	7
159-F_3-Phe	20,600 ± 2100*	1.3 ± 0.2	0.1 ± 0.01	5
207-Phe (WT)	559 ± 30	1.7 ± 0.2	0.8 ± 0.1	3
207-F-Phe	615 ± 54	1.6 ± 0.2	0.5 ± 0.1	5
207-F_2-Phe	32,300 ± 1500*	2.1 ± 0.2	0.1 ± 0.03	4
207-CN-Phe	5080 ± 140*	1.6 ± 0.1	0.4 ± 0.02	7
207-F_3-Phe	47,900 ± 2100*	1.9 ± 0.4	0.1 ± 0.03	4

* $P < 0.05$, significantly different from WT for EC_{50} values (Student's t test).

TABLE 3

Taurine dose-response data for GlyRs with unnatural amino acids incorporated at positions 63, 159, 161, and 207

Data are presented as mean ± S.E.M.

	EC_{50}	n_H	$R_{(\text{Taurine/Gly})}$	n
	μM			
159-Phe (WT)	507 ± 29	2.1 ± 0.3	1.0 ± 0.05	5
159-F-Phe	681 ± 56	2.1 ± 0.2	0.7 ± 0.04	5
159-CN-Phe	5100 ± 458*	1.4 ± 0.1	0.7 ± 0.04	5
159-F_3-Phe	9700 ± 2400*	1.9 ± 0.4	0.03 ± 0.005	5
207-F-Phe	720 ± 89	1.2 ± 0.4	0.6 ± 0.10	6
207-F_2-Phe	5600 ± 430*	0.8 ± 0.2	0.03 ± 0.005	3
207-F_3-Phe	3600 ± 230*	0.7 ± 0.4	0.004 ± 0.001	4
63-F-Phe	389 ± 41	1.2 ± 0.1	0.6 ± 0.03	6
63-F_2-Phe	N.R.			8
161-F-Phe	2500 ± 200*	1.6 ± 0.2	0.6 ± 0.03	6
161-CN-Phe	423 ± 14	1.4 ± 0.1	0.7 ± 0.04	7

N.R., no response at 100 mM.

* $P < 0.05$, significantly different from WT for EC_{50} values (Student's t test).

interaction with taurine. Thus, 4-F-Phe, 4-CN, and 3,4,5-F_3-Phe caused ~1.3-, 10-, and ~19-fold increases in EC_{50}, respectively (Table 3; Fig. 2F). A plot of relative log EC_{50} values and the cation-π binding ability of the incorporated Phe derivative indicate a cation-π interaction between taurine and Phe159 (Fig. 3B). The slope of the linear fit for taurine is less than that for β-alanine (Fig. 3C), suggesting a weaker interaction here.

Taurine Effects at Tyr161 Mutant Receptors. Previous data suggest that Tyr161 may participate in agonist binding at the GlyR: its substitution with Phe, combined with an F159Y mutation, resulted in a receptor that was ~100-

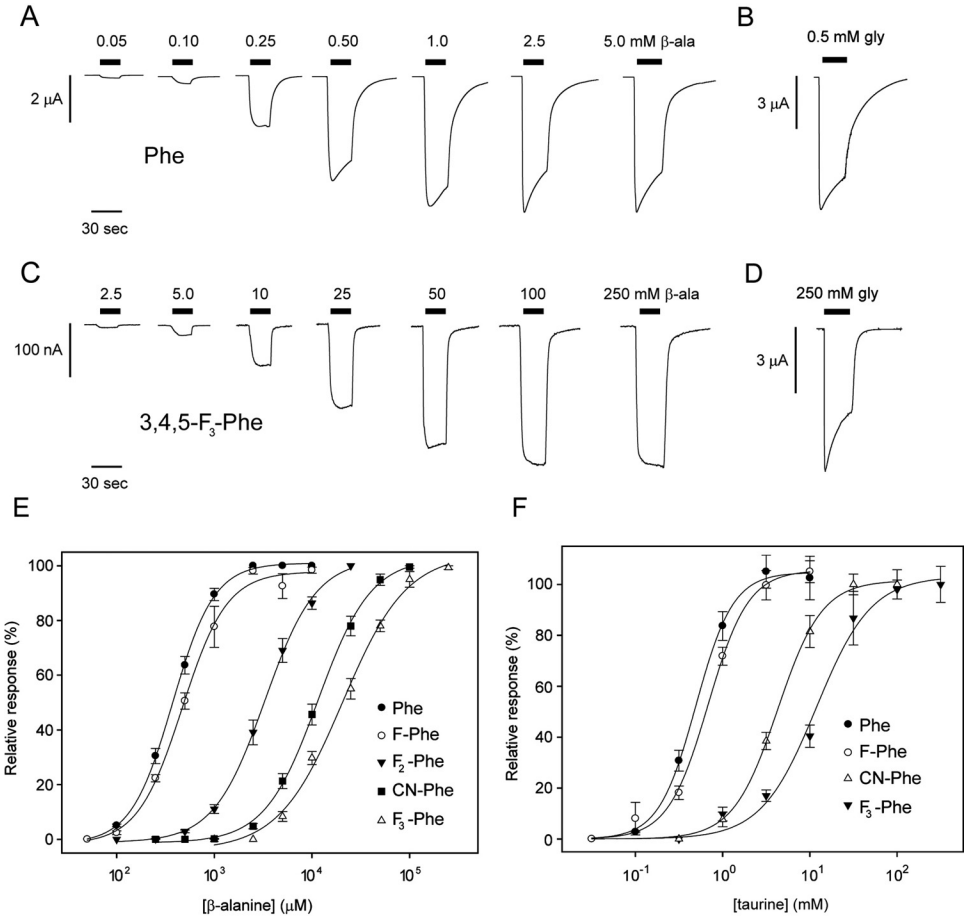

Fig. 2. Successive fluorination leads to monotonic decreases in β-alanine and taurine sensitivity at position 159. β-Alanine-induced current traces recorded from oocytes injected with GlyR Phe159TAG mRNA and Phe tRNA (A) or GlyR Phe159TAG mRNA and 3,4,5-F_3-Phe tRNA (C). Glycine-induced current traces in B and D are shown for comparison. Horizontal bars indicate duration of agonist application. E and F, concentration-response curves for β-alanine- and taurine-induced currents, respectively.

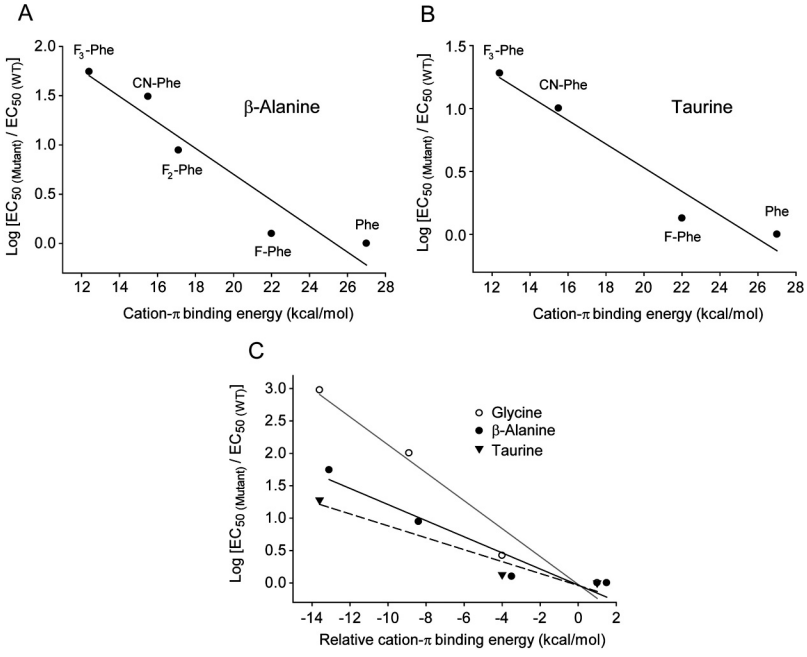

Fig. 3. GlyR responses from Phe159 mutant receptors yield linear cation-π plots for both β-alanine and taurine. A and B, the log EC_{50} (based on data in Tables 2 and 3) normalized to the WT log EC_{50} was used to generate a plot of the GlyR Phe159 mutants for β-alanine (A) and taurine (B). The linear fits were $y = 3.34 - 0.13x$ ($r = 96$) for β-alanine and $y = 2.41 - 0.09x$ ($r = 0.97$) for taurine. C, cation-π plots comparing the cation-π interactions for glycine, β-alanine, and taurine at the Phe159 GlyR (glycine data from Pless et al., 2008). For better comparison, the x values are offset so all linear fits converge at zero. Note that the value for CN-Phe is omitted in the gradient for β-alanine and taurine. This did not change the slope of the linear fit significantly [for comparison, the linear fit for glycine (without CN-Phe) is $y = 5.58 - 0.22x$ ($r = 0.99$)].

fold more highly sensitive to β-alanine and ~30-fold more sensitive to taurine (Schmieden et al., 1993). The β-alanine effect has been shown to be indirect (De Saint Jan et al., 2001), but the increase in taurine EC_{50} (~5 fold) with 4-F-Phe at this position suggests a possible cation-π interaction. Substitution with 4-CN-Phe, however, resulted in a taurine EC_{50} similar to that in WT receptors, indicating there is no cation-π interaction here (Table 3).

Characterization of the Phe207TAG Construct. 4-F-Phe substitution at position 207 did not change the β-alanine EC_{50} compared with WT (Table 2). Incorporation of 3,4-F_2-Phe and 3,4,5-F_3-Phe resulted in large increases in the β-alanine EC_{50} (60- and 90-fold, respectively), although substitution with 4-CN-Phe produced only a 9-fold increase in EC_{50}; these data are inconsistent with a cation-π interaction. The pattern was broadly similar for taurine, with no change in the EC_{50} with 4-F-Phe, but some increases when 3,4-F_2-Phe and 3,4,5-F_3-Phe were incorporated (11- and 7-fold, respectively; Table 3). These data also do not support a cation-π interaction at position 207.

Compared with the maximum currents observed with Phe207 mutants for glycine (similar to wild type), R_{max} values were much reduced when these mutants were activated by β-alanine or taurine (Tables 2 and 3). Changes in R_{max} values may indicate an effect on receptor gating, so Phe207 may play a different role in gating for these partial agonists than for the full agonist glycine.

Locating β-Alanine in the Binding Pocket. We have previously created a model of the glycine receptor binding pocket, and docking of glycine revealed the potential for a strong cation-π interaction with Phe159 (Pless et al., 2008) (Fig. 4, B and C). Here we have docked β-alanine into the same model, and the data show that it is located in a broadly similar orientation to glycine, but the amino group is further from the center of Phe159 than the amino group of glycine (Fig. 4, D and E).

Locating Taurine in the Binding Pocket. We also docked taurine into the ligand binding pocket, and the results reveal that its amino group, like that of β-alanine, is further from Phe159 than the amino group of glycine. However, the major difference compared with glycine is the location of the sulfonate group, which is close to Phe63 (Fig. 4, F and G). This location is supported by the mutagenesis data, which suggest that taurine is more sensitive than glycine and β-alanine to changes in this residue (Tables 1 and 3).

Discussion

In this study, we demonstrate that the GlyR partial agonists β-alanine and taurine can form cation-π interactions with Phe159 in the binding pocket of the glycine receptor. For both ligands, there is a clear correlation between the EC_{50} and the cation-π binding ability of phenylalanine derivatives incorporated at position 159 in the GlyR (Fig. 3), making a strong argument for cation-π interactions between the amino groups of β-alanine and taurine and this loop B phenylalanine. The data do not support a cation-π interaction between either taurine or β-alanine and Phe63 or Phe207. The identification of single cation-π interactions at Phe159 with both these agonists is consistent with an earlier study that also demonstrated a single cation-π interaction between the amino group of the principal GlyR agonist glycine and Phe159 (Pless et al., 2008). The data also conform to the observations that the most common cation-π interactions in Cys-loop receptors are mediated through aromatic amino acids in loop B (Trp149 of the nAChR, Trp183 of the 5-HT_3 receptor, Tyr198 of the $GABA_C$ receptor, and Phe159 of the GlyR), although cation-π interactions with residues on loop A

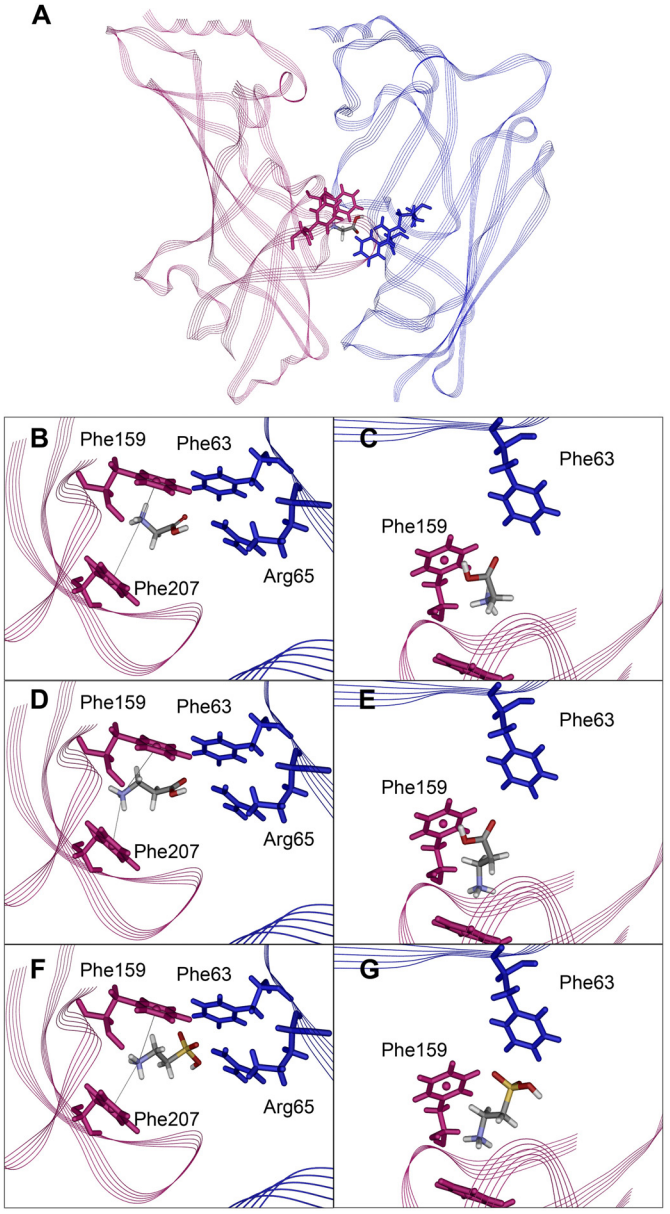

Fig. 4. Docking of β-alanine and taurine into the GlyR binding pocket. A, location of an agonist in the binding pocket. Model of the extracellular domain of the GlyR showing two of the five subunits and some of the residues (in stick form) that surround the agonist (Corey/Pauling/Koltun coloring) in the binding pocket: Phe159 and Phe207 are located on the principal (red) subunit, whereas Phe63 and Arg65 are located on the complementary (blue) subunit. B and C, two views of glycine docked into the binding site from different perspectives showing the amino group located between Phe159 and Phe207 and the carboxyl group some distance from Phe63. D and E, two views of β-alanine docked into the binding site pocket showing that the amino group is further from Phe159 compared with the amino group of glycine, although the carboxyl group is similar distance from Phe63. F and G, two views of taurine docked into the binding pocket showing that the amino group is again further from Phe159 compared with glycine, and the sulfonate group is closer to Phe63 than the carboxyl of glycine.

(Tyr97 in the $\alpha1\beta2$ $GABA_A$ receptor) and loop C (Trp226 on the MOD-1 receptor) have also been reported (Zhong et al., 1998; Beene et al., 2002; Mu et al., 2003; Lummis et al., 2005; Padgett et al., 2007; Pless et al., 2008).

An intriguing aspect of our new data is the fact that the slopes of the fluorination plots for β-alanine and taurine are significantly reduced compared with that for glycine (Fig. 3C). This suggests that the relative strength of the cation-π interactions is glycine $>$ β-alanine $>$ taurine, as weakening of the cation-π interaction by successive fluorination has the most dramatic effect on activation by glycine, then β-alanine, and finally taurine. The slope for glycine (Pless et al., 2008) resembles that of other agonists containing a primary amine, such as GABA (Padgett et al., 2007), whereas the slopes for β-alanine and taurine are more similar to that of the quaternary ammonium ion of acetylcholine (β-alanine = -0.13; taurine = -0.09; acetylcholine = -0.10) (Zhong et al., 1998). It has been suggested previously that the less focused charge of the quaternary ammonium ion of acetylcholine leads to a weaker cation-π interaction compared with agonists with a primary amine, such as glycine, serotonin, and GABA (Beene et al., 2002; Pless et al., 2008). Here, however, we show that agonists with primary amines (β-alanine and taurine) can also form cation-π interactions that are similar to those formed by agonists with a quaternary ammonium. This seems to suggest that not only the nature of the cationic moiety but also the exact orientation determines the strength of a cation-π interaction at a given site. Such an idea is consistent with a computational study, which demonstrated that the exact orientation of the aromatic residue and the cation are crucial for a strong cation-π interaction (Gallivan and Dougherty, 1999).

To further explore this hypothesis, we examined the possible locations of the agonists in a model of the GlyR binding site. The only structural difference between glycine and β-alanine is the slightly elongated carbonyl backbone of β-alanine (Fig. 1A). Docking studies locate the amino group of β-alanine further from the center of the aromatic ring of Phe159, which would result in a weaker cation-π interaction (Fig. 4).

Like β-alanine, taurine also has an elongated carbonyl backbone and a sulfonate group in place of the carboxyl group (Fig. 1A). Ligand docking shows that this molecule is also oriented with the amino group further displaced from Phe159 than it is for glycine, and it is in a different orientation to β-alanine, providing an explanation for the weaker cation-π interaction. It is possible, however, that the model is not quite accurate for partial agonists, resulting in inaccuracies in docking; a recent crystallographic study revealed that partial agonists bind to a more open binding pocket in acetylcholine binding protein because of the reduced closure of the C loop (Hibbs et al., 2009). It is noteworthy, therefore, that substitutions of Phe207 in loop C had severe effects on β-alanine- and taurine-induced currents (taurine $>$ β-alanine) but little effect on glycine-induced currents. Taken together, these results suggest that glycine, β-alanine, and taurine may each induce distinct conformational changes in and around the GlyR binding site (taurine $<$ β-alanine $<$ glycine), an idea that is also supported by a recent study using fluorescent reporter groups (Pless and Lynch, 2009).

These different conformational changes could possibly be the cause of the different magnitudes of the responses produced by these different agonists (i.e., their efficacy). Intrinsic efficacy is related to agonist concentration, agonist affinity, and receptor number (Furchgott, 1966) but also depends on other factors, including the receptor state (e.g., open, closed, desensitized) and the kinetics of the interactions between these states (Colquhoun and Hawkes, 1987). In addition, there are contributions from more "external" factors, such as the expression system or brain region; taurine, for example, has different efficacies in oocytes and human embryonic kidney cells, and also in brain and spinal cord neurons (Killcross et al., 1997; Farroni and McCool, 2004). Therefore, it is not surprising that a quantitative understanding of the link between the receptor-ligand interactions and efficacy is still in its infancy. We are tempted to speculate that the different strengths of the different cation-π interactions with the different agonists are related to their different efficacies. A lower efficacy could be the result of reduced loop C closure, as a result of a decrease in cation-π binding energy, which could result in less efficient receptor activation. This may be reflected in—or caused by—the decrease in desensitization rates that we observed, which indicates a change in the kinetics of the interactions between the different states. Considerable work is needed to prove or disprove this speculative hypothesis.

In principle, aromatic amino acids other than Phe159 could also contribute to a cation-π interaction in the GlyR binding site. Phe99, Phe100, Tyr161, and Tyr202 are located close to the binding site, but previous studies have suggested that they are unlikely to contribute to a cation-π interaction. Substituting Phe99 or Phe100 with a small and nonaromatic amino acid, alanine, resulted in only a minor shift in the EC_{50} values (Vafa et al., 1999). A pioneering study suggested that Tyr161 was important for β-alanine binding (Schmieden et al., 1993), but more recent work convincingly demonstrated that this residue has only indirect effects (De Saint Jan et al., 2001). The present study also indicates that there is no cation-π interaction between this residue and taurine. Finally, two studies have demonstrated that the hydroxyl group of Tyr202, rather than its aromatic character, is crucial for agonist binding (Rajendra et al., 1995a; Grudzinska et al., 2005).

It is also conceivable that the cation originates from a positively charged amino acid near the binding site, rather than the amino group of the ligand: Arg65, Arg131, Lys200, and Lys206 are all in close physical proximity to the binding site. However, neutralizing Arg131, Lys200, or Lys206 results in only minor shifts in agonist EC_{50} values (Vandenberg et al., 1992; Yang et al., 2007), whereas Arg65 interacts with the negatively charged head group of both full and partial agonists (Grudzinska et al., 2005). We thus conclude that the amino groups of β-alanine and taurine form cation-π interactions with Phe159.

GlyRs are emerging as pharmacological targets, and thus the exact binding mechanism of full agonists, partial agonistsb and even antagonists in GlyRs is of particular interest (Laube et al., 2002). The results presented here will aid future efforts to delineate the exact binding mode of these compounds and may be useful to predict (and control) agonist efficacy in Cys-loop receptors.

Authorship Contributions

Participated in research design: Pless, Lynch, Lester, Dougherty, and Lummis.

Conducted experiments: Pless, Hanek, Price, and Lummis.

Contributed new reagents or analytic tools: Dougherty.

Performed data analysis: Pless, Hanek, Price, and Lummis.

Wrote or contributed to the writing of the manuscript: Pless, Hanek, Price, Lynch, Lester, Dougherty, and Lummis.

Other: Lynch, Lester, Dougherty, and Lummis acquired funding.

References

Beene DL, Brandt GS, Zhong W, Zacharias NM, Lester HA, and Dougherty DA (2002) Cation-pi interactions in ligand recognition by serotonergic (5-HT3A) and nicotinic acetylcholine receptors: the anomalous binding properties of nicotine. *Biochemistry* **41:**10262–10269.

Beene DL, Dougherty DA, and Lester HA (2003) Unnatural amino acid mutagenesis in mapping ion channel function. *Curr Opin Neurobiol* **13:**264–270.

Beene DL, Price KL, Lester HA, Dougherty DA, and Lummis SC (2004) Tyrosine residues that control binding and gating in the 5-hydroxytryptamine3 receptor revealed by unnatural amino acid mutagenesis. *J Neurosci* **24:**9097–9104.

Bocquet N, Nury H, Baaden M, Le Poupon C, Changeux JP, Delarue M, and Corringer PJ (2009) X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. *Nature* **457:**111–114.

Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, and Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411:**269–276.

Colquhoun D and Hawkes AG (1987) A note on correlations in single ion channel records. *Proc R Soc Lond B Biol Sci* **230:**15–52.

De Saint Jan D, David-Watine B, Korn H, and Bregestovski P (2001) Activation of human alpha1 and alpha2 homomeric glycine receptors by taurine and GABA. *J Physiol* **535:**741–755.

Dougherty DA (2000) Unnatural amino acids as probes of protein structure and function. *Curr Opin Chem Biol* **4:**645–652.

Dougherty DA (2008) Cys-loop neuroreceptors: structure to the rescue? *Chem Rev* **108:**1642–1653.

Farroni JS and McCool BA (2004) Extrinsic factors regulate partial agonist efficacy of strychnine-sensitive glycine receptors. *BMC Pharmacol* **4:**16.

Furchgott RF (1966) Metabolic factors that influence contractility of vascular smooth muscle. *Bull N Y Acad Med* **42:**996–1006.

Gallivan JP and Dougherty DA (1999) Cation-pi interactions in structural biology. *Proc Natl Acad Sci USA* **96:**9459–9464.

Grudzinska J, Schemm R, Haeger S, Nicke A, Schmalzing G, Betz H, and Laube B (2005) The beta subunit determines the ligand binding properties of synaptic glycine receptors. *Neuron* **45:**727–739.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schütz B, Abo-Salem OM, et al. (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* **304:**884–887.

Hibbs RE, Sulzenbacher G, Shi J, Talley TT, Conrod S, Kem WR, Taylor P, Marchot P, and Bourne Y (2009) Structural determinants for interaction of partial agonists with acetylcholine binding protein and neuronal alpha7 nicotinic acetylcholine receptor. *EMBO J* **28:**3040–3051.

Hilf RJ and Dutzler R (2008) X-ray structure of a prokaryotic pentameric ligand-gated ion channel. *Nature* **452:**375–379.

Hilf RJ and Dutzler R (2009) Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. *Nature* **457:**115–118.

Kearney PC, Nowak MW, Zhong W, Silverman SK, Lester HA, and Dougherty DA (1996) Dose-response relations for unnatural amino acids at the agonist binding site of the nicotinic acetylcholine receptor: tests with novel side chains and with several agonists. *Mol Pharmacol* **50:**1401–1412.

Killcross S, Robbins TW, and Everitt BJ (1997) Different types of fear-conditioned behaviour mediated by separate nuclei within amygdala. *Nature* **388:**377–380.

Lape R, Colquhoun D, and Sivilotti LG (2008) On the nature of partial agonism in the nicotinic receptor superfamily. *Nature* **454:**722–727.

Laube B, Maksay G, Schemm R, and Betz H (2002) Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? *Trends Pharmacol Sci* **23:**519–527.

Le Novère N and Changeux JP (2001) The Ligand Gated Ion Channel database: an example of a sequence database in neuroscience. *Philos Trans R Soc Lond B Biol Sci* **356:**1121–1130.

Legendre P (2001) The glycinergic inhibitory synapse. *Cell Mol Life Sci* **58:**760–793.

Lewis TM, Schofield PR, and McClellan AM (2003) Kinetic determinants of agonist action at the recombinant human glycine receptor. *J Physiol* **549:**361–374.

Lummis SC, Beene DL, Harrison NJ, Lester HA, and Dougherty DA (2005) A cation-pi binding interaction with a tyrosine in the binding site of the GABAC receptor. *Chem Biol* **12:**993–997.

Lynch JW (2004) Molecular structure and function of the glycine receptor chloride channel. *Physiol Rev* **84:**1051–1095.

Mecozzi S, West AP Jr, and Dougherty DA (1996) Cation-pi interactions in aromatics of biological and medicinal interest: electrostatic potential surfaces as a useful qualitative guide. *Proc Natl Acad Sci USA* **93:**10566–10571.

Miyazawa A, Fujiyoshi Y, and Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423:**949–955.

Mu TW, Lester HA, and Dougherty DA (2003) Different binding orientations for the same agonist at homologous receptors: a lock and key or a simple wedge? *J Am Chem Soc* **125:**6850–6851.

Nowak MW, Gallivan JP, Silverman SK, Labarca CG, Dougherty DA, and Lester HA (1998) In vivo incorporation of unnatural amino acids into ion channels in *Xenopus* oocyte expression system. *Methods Enzymol* **293:**504–529.

Padgett CL, Hanek AP, Lester HA, Dougherty DA, and Lummis SC (2007) Unnatural amino acid mutagenesis of the GABA(A) receptor binding site residues reveals a novel cation-pi interaction between GABA and beta 2Tyr97. *J Neurosci* **27:**886–892.

Pless SA, Dibas MI, Lester HA, and Lynch JW (2007) Conformational variability of the glycine receptor M2 domain in response to activation by different agonists. *J Biol Chem* **282:**36057–36067.

Pless SA and Lynch JW (2009) Magnitude of a conformational change in the glycine receptor beta1-beta2 loop is correlated with agonist efficacy. *J Biol Chem* **284:**27370–27376.

Pless SA, Millen KS, Hanek AP, Lynch JW, Lester HA, Lummis SC, and Dougherty DA (2008) A cation-pi interaction in the binding site of the glycine receptor is mediated by a phenylalanine residue. *J Neurosci* **28:**10937–10942.

Rajendra S, Lynch JW, Pierce KD, French CR, Barry PH, and Schofield PR (1995a) Mutation of an arginine residue in the human glycine receptor transforms beta-alanine and taurine from agonists into competitive antagonists. *Neuron* **14:**169–175.

Rajendra S, Vandenberg RJ, Pierce KD, Cunningham AM, French PW, Barry PH, and Schofield PR (1995b) The unique extracellular disulfide loop of the glycine receptor is a principal ligand binding element. *EMBO J* **14:**2987–2998.

Reeves DC, Sayed MF, Chau PL, Price KL, and Lummis SC (2003) Prediction of 5-HT3 receptor agonist-binding residues using homology modeling. *Biophys J* **84:**2338–2344.

Schmieden V and Betz H (1995) Pharmacology of the inhibitory glycine receptor: agonist and antagonist actions of amino acids and piperidine carboxylic acid compounds. *Mol Pharmacol* **48:**919–927.

Schmieden V, Kuhse J, and Betz H (1992) Agonist pharmacology of neonatal and adult glycine receptor alpha subunits: identification of amino acid residues involved in taurine activation. *EMBO J* **11:**2025–2032.

Schmieden V, Kuhse J, and Betz H (1993) Mutation of glycine receptor subunit creates beta-alanine receptor responsive to GABA. *Science* **262:**256–258.

Thompson AJ, Lochner M, and Lummis SC (2007) The anitmalarial drugs quinine, chloroquine and mefloquine are antagonists at 5-HT3 receptors. *Br J Pharmacol* **151:**666–677.

Thompson AJ, Price KL, Reeves DC, Chan SL, Chau PL, and Lummis SC (2005) Locating an antagonist in the 5-HT3 receptor binding site using modeling and radioligand binding. *J Biol Chem* **280:**20476–20482.

Unwin N (1995) Acetylcholine receptor channel imaged in the open state. *Nature* **373:**37–43.

Vafa B, Lewis TM, Cunningham AM, Jacques P, Lynch JW, and Schofield PR (1999) Identification of a new ligand binding domain in the alpha1 subunit of the inhibitory glycine receptor. *J Neurochem* **73:**2158–2166.

Vandenberg RJ, Handford CA, and Schofield PR (1992) Distinct agonist- and antagonist-binding sites on the glycine receptor. *Neuron* **9:**491–496.

Weiner SJ, Kollman PA, Case DA, Singh C, Ghio G, Alagona S, Profeta P, and Weiner P (1984) A new force field for molecular mechanical simulation of nucleic acids and proteins. *J Am Chem Soc* **106:**765–784.

Xiu X, Puskar NL, Shanata JA, Lester HA, and Dougherty DA (2009) Nicotine binding to brain receptors requires a strong cation-pi interaction. *Nature* **458:**534–537.

Yang Z, Ney A, Cromer BA, Ng HL, Parker MW, and Lynch JW (2007) Tropisetron modulation of the glycine receptor: femtomolar potentiation and a molecular determinant of inhibition. *J Neurochem* **100:**758–769.

Zhong W, Gallivan JP, Zhang Y, Li L, Lester HA, and Dougherty DA (1998) From ab initio quantum mechanics to molecular neurobiology: a cation-pi binding site in the nicotinic receptor. *Proc Natl Acad Sci USA* **95:**12088–12093.

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