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A CATION-π INTERACTION AT A PHENYLALANINE RESIDUE IN THE GLYCINE RECEPTOR BINDING SITE IS CONSERVED FOR DIFFERENT AGONISTS

Stephan A. Pless; Ariele P. Hanek; Kerry. L. Price; Joseph W. Lynch; Henry A. Lester; Dennis A. Dougherty and Sarah C.R. Lummis

School of Biomedical Sciences and Queensland Brain Institute, University of Queensland, Brisbane, Queensland 4072 Australia (S.A.P., J.W.L); Division of Chemistry and Chemical Engineering (A.P.L., D.A.D.) and Biology (H.A.L), California Institute of Technology, Pasadena, California 91125 USA; Department of Biochemistry, University of Cambridge, Cambridge UK (K.L.P., S.C.R.L). Current address S.A.P.: Department of Anaesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, BC V6T 1Z3, Canada.

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Running title: A conserved glycine receptor cation-π interaction

*__Author for correspondence__:

SCRL: Department of Biochemistry,

Tennis Court Road, Cambridge CB2 1QW.

Tel: (+44)1223 765950 Fax (+44)1223 333345

Email: sl120@cam.ac.uk

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Abbreviations: AChBP: acetylcholine binding protein; AChR: acetylcholine receptor; GlyR: glycine receptor; nAChR: Nicotinic acetylcholine receptor; 5-HT_3R: 5-hydroxytryptamine receptor

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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 are conserved among Cys-loop receptors. Similarly, 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 previously demonstrated that Phe159 in the glycine receptor (GlyR) α1 subunit forms a strong cation-π interaction with the principal agonist, glycine. In this 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. Modelling 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.

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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 the generation of motor rhythm, coordination of reflex circuits, and the processing of sensory signals (Harvey et al., 2004; Legendre, 2001). Along with $GABA_A$ receptors ($GABA_A$Rs), 5-hydroxytryptamine$_3$ receptors (5-HT_3Rs) and nicotinic acetylcholine receptors (nAChRs), GlyRs belong to the Cys-loop receptor family (Le Novere and Changeux, 2001). All Cys-loop members are comprised of five subunits that are pseudosymmetrically arranged around a central ion-conducting pore. Individual subunits consist of a large N-terminal ligand-binding domain (LBD) and four transmembrane helices that are connected by loops of varying size (Miyazawa et al., 2003; Unwin, 1995). Structural studies on acetylcholine binding proteins (AChBPs) (Brejc et al., 2001) and prokaryotic Cys-loop receptor homologues (Bocquet et al., 2009; Hilf and Dutzler, 2008; 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 previously shown 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). Similarly, 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 (Beene et al., 2002; Dougherty, 2008; Lummis et al., 2005; Mu et al., 2003; Padgett et al., 2007; Zhong et al., 1998). 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; Dougherty, 2008; Mu et al., 2003; 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 (Lewis et al., 2003; Schmieden et al., 1992). β-Alanine and taurine are structurally similar to glycine (Fig. 1A) and both agonists compete for the same binding site (Schmieden and Betz, 1995; Schmieden et al., 1992) According to a single channel kinetic analysis, dissociation rates for β-alanine and taurine are increased 2.5 fold and 4 fold, respectively (compared to glycine), and efficacy 2 fold and 5 fold (Lewis et al., 2003). To explore the molecular mechanism(s) involved in these differences we used the nonsense suppression methodology (Beene et al., 2003; Dougherty, 2000) to incorporate unnatural amino acids into the GlyR binding site to determine if β-alanine and taurine, like glycine, form a cation-π interaction with a phenylalanine residue at position 159.

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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 QuickChange mutagenesis kit (Stratagene, La Jolla, CA) was used for site-directed mutagenesis. Successful incorporation of mutations was confirmed by automated sequencing. Capped cRNA for oocyte injection was generated using the mMessage mMachine kit (Ambion, Austin, TX). *Xenopus laevis* (Nasco, WI). Oocytes were prepared as previously described (Pless et al., 2007), and injected with cRNA alone or cRNA plus tRNA (see below). 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 previously described (Beene et al., 2004). In short, unnatural amino acids (as shown in Figure 1) were chemically synthesized as NVOC (nitroveratryloxycarbonyl)-protected cyanomethyl esters and coupled to the dinucleotide dCA. The resulting product was subsequently ligated enymatically to a 74-mer THG73 $tRNA_{CUA}$ as described (Nowak et al., 1998). The aminoacyl tRNA was deprotected by photolysis (Kearney et al., 1996) directly prior to co-injection with the mRNA. Typically, 10 to 15 ng of mRNA were co-injected with 20 to 30 ng of tRNA-amino acid in a total volume of 50 nl mRNA containing the TAG stop codons was injected alone or together with tRNA-dCA (with no amino acid attached) in control experiments. 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.

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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 glycine or β-alanine. All recordings were performed at 22-25°C. Glycine and β-alanine (Sigma, St. Louis, MO) were stored at -20°C as 1 M and 500 mM aliquots, respectively. Both were diluted in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4) directly prior to 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 non-linear 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 = 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 (Sali and Blundell, 1993) based on the crystal structure of AChBP in the agonist bound state (carbomylcholine, PDB ID; 1uv6). The model was energy minimized in SYBYL version 6.8 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. Docking was performed using GOLD 3.0 (The Cambridge Crystallographic Data Centre, Cambridge, UK) as previously described (Thompson et al., 2007; Thompson and Lummis, 2007) with the ligands constrained to dock within 4.5Å of Arg65, as 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).

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Results

Locating the residues of interest using conventional mutagenesis. In this study, we chose to focus on Phe63, Phe159 and Phe207. Mutations at these sites markedly affect receptor function, and aligning residues in other receptors form cation-π interactions in previous studies (Grudzinska et al., 2005; Rajendra et al., 1995b; Pless et al., 2008). Phe63Ala substitution caused increases in EC_{50}s of 760, 280 and 360 fold for glycine, β-alanine and taurine, while Phe63Tyr 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 size here is critical for taurine binding/activation (Table 1).

Phe159Tyr substitution decreased EC_{50}s for all three agonists as previously reported (Schmieden et al., 1993) while Phe159Ala substitution caused large increases (70-260 fold) (Table 1).

Phe207Ala substitution eliminated all agonist responses. Phe207Tyr had no significant effect on EC_{50}s. 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).

Characterisation of the Phe63TAG construct. Incorporating a Phe at position 63 with the nonsense suppression method resulted in functional receptors with EC_{50}s similar to 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 co-injection of Phe63TAG mRNA and Phe tRNA. A single fluorination (4-F-Phe) at position 63 resulted in a three or two-fold decrease in EC_{50} for β-alanine or taurine respectively (Tables 2 & 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-fold and 5-fold increases in the β-alanine EC_{50}, respectively (compared to

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WT). As the EC_{50}s 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}s 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 2A, C and E; Table 2). These results strongly suggest a cation-π interaction between β-alanine and Phe159. The data include not only fluorinated analogues, but also a Phe derivative with a cyano group 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. 2B and D) with β-alanine-induced currents (Fig. 2A and C) reveals that 3,4,5-F_3-Phe, but not Phe, markedly reduces receptor desensitisation 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 interaction with taurine. Thus 4-F-Phe caused a small (~1.3 fold) increase in EC_{50}, with 4-CN causing an increase of ~ 10 fold, and 3,4,5-F_3-Phe an increase of ~19 fold (Table 3 and Fig. 2F). A plot of relative log EC_{50}s and the cation-π binding ability of the incorporated Phe derivative

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these data 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 a Phe159Tyr mutation, resulted in a receptor that was ~100 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 suggesting a possible cation-π interaction. Substitution with 4-CN-Phe, however, resulted in a taurine EC_{50} similar to WT receptors, indicating there is no cation-π interaction here (Table 3).

Characterisation of the Phe207TAG construct. 4-F-Phe substitution at position 207 did not change the β-alanine EC_{50} compared to 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 only 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.

In comparison to 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.

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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. 4B/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 centre of Phe159 than the amino group of glycine (Fig. 4D/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 to glycine is the location of the sulphonate group, which is close to Phe63 (Fig. 4F/G). This location is supported by the mutagenesis data, which suggest taurine is more sensitive than glycine and β-alanine to changes in this residue (Tables 1 & 3).

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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 (Trp 149 of the nAChR; Trp 183 of the 5-HT_3R, Tyr 198 of the $GABA_C$R and Phe159 of the GlyR; Beene et al., 2002; Lummis et al., 2005; Pless et al., 2008; Zhong et al., 1998), although cation-π interactions with residues on loop A (Tyr97 in the α1β2 $GABA_A$R) and loop C (Trp226 on the MOD-1R) have also been reported (Mu et al., 2003; Padgett et al., 2007).

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 to 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,

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such as GABA (Pless et al., 2008), while 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 previously suggested that the less focussed charge of the quaternary ammonium ion of acetylcholine leads to a weaker cation-π interaction compared to 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 it is not only the nature of the cationic moiety but also the exact orientation that 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 centre 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 sulphonate 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 however possible the model is not quite accurate for partial agonists,

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resulting in inaccuracies in the docking; a recent crystallographic study revealed partial agonists bind to a more open binding pocket in AChBP, due to reduced closure of the C loop (Hibbs et al., 2009). It is therefore interesting to note 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 is also dependent on other factors including the receptor state (e.g. open, closed, desensitised) 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 HEK cells, and also in brain and spinal cord neurones (Farroni and McCool, 2004; Killcross et al. 1997). 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, due to 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

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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 non-aromatic amino acid, alanine, resulted in only a minor shift in the EC_{50}s (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 indirectly 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 it is the hydroxyl group of Tyr202 and not its aromatic character that is crucial for agonist binding (Grudzinska et al., 2005; Rajendra et al., 1995a).

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, neutralising Arg131, Lys200 or Lys206 results in only minor shifts in agonist EC_{50}s (Vandenberg et al., 1992; Yang et al., 2007), while Arg65 interacts with the negatively charged head group of both full and partial agonists (Grudzinska et al., 2005). We thus conclude that it is the amino groups of β-alanine and taurine that form cation-π interactions with Phe159.

GlyRs are emerging as pharmacological targets, and thus the exact binding mechanism of full agonists, partial agonists and even antagonists in GlyRs is of particular interest (Laube

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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.

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Authorship Contributions

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

Conducted experiments: Pless, Lummis, Price, Hanek.

Contributed new reagents or analytic tools: Dougherty.

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

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

Other: Dougherty, Lummis, Lynch, Lester acquired funding for the research.

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Footnotes

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Figure Legends

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

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 (reproduced from Pless et al., 2008). Horizontal bars indicate duration of agonist application. (E) / (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) / (B) The log EC_{50} (based on data in Table 2 and 3) normalised 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 reproduced 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

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comparison, the linear fit for glycine (without CN-Phe) is $y = 5.58 – 0.22x$ ($r = 0.99$) (Pless et al., 2008).

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 stock form) that surround the agonist (CPK colouring) in the binding pocket: Phe159 and Phe207 are located on the principal (red) subunit, while Phe63 and Arg65 are located on the complementary (blue) subunit. (B) / (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) / (E) Two views of β-alanine docked into the binding site pocket showing that the amino group is further from Phe159 when compared to the amino group of glycine, although the carboxyl group is similar distance from Phe63. (F) / (G) Two vies of taurine docked into the binding pocket showing that the amino group is again further from Phe159, when compared to glycine, and the sulphonate group is closer to Phe63 than the carboxyl of glycine.

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Table 1: Dose-response data for GlyRs with conventional mutations at positions 63, 159 and 207.

	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	NR
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}	NR	NR	NR
R_{max}		-	-
F207Y EC_{50}	161 ± 23	520 ± 88	754 ± 122
R_{max}		0.6 ± 0.06	0.7 ± 0.04

EC_{50} = value for half-maximal activation; R_{max} = ratio of maximal currents induced by β-alanine or taurine vs. glycine. Data = mean ± S.E.M, n=3-8. NR = No response at 100mM .

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Table 2: β-Alanine dose-response data for GlyRs with unnatural amino acids incorporated at positions 63, 159 and 207.

	EC_{50} (μM)	n_H	$R_{(\beta\text{-ala/gly})}$	n
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	11500 ± 500 *	1.6 ± 0.1	0.7 ± 0.1	7
159-F_3 -Phe	20600 ± 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	32300 ± 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	47900 ± 2100 *	1.9 ± 0.4	0.1 ± 0.03	4

Data = mean ± S.E.M. Asterisks indicate significant differences to WT for EC_{50}s (* = $p < 0.05$; Student's t-test).

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Table 3: Taurine dose-response data for GlyRs with unnatural amino acids incorporated at positions 63, 159, 161 and 207.

	EC_{50} (µM)	n_H	$R_{(tau/gly)}$	n
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	NR	-	-	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

Data = mean ± S.E.M. Asterisks indicate significant differences to WT for EC_{50}s (* = $p < 0.05$; Student's t-test); NR= no response at 100mM.

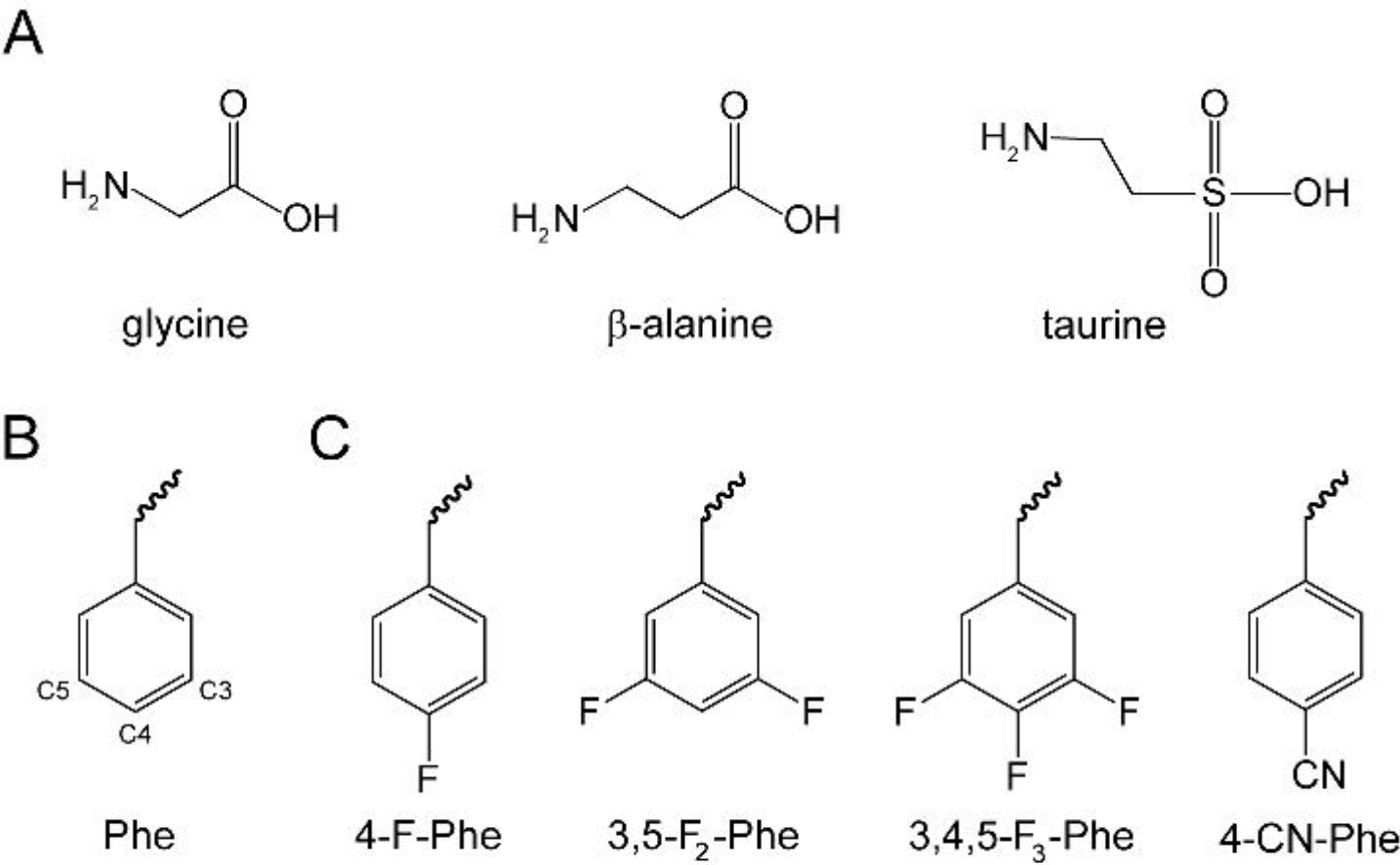

A
O
H2N
OH
glycine
O
H2N
OH
β-alanine
O
H2N
S
OH
O
taurine
B
C5
C3
C4
Phe
C
F
4-F-Phe
F
F
3,5-F2-Phe
F
F
F
3,4,5-F3-Phe
CN
4-CN-Phe

Figure 1

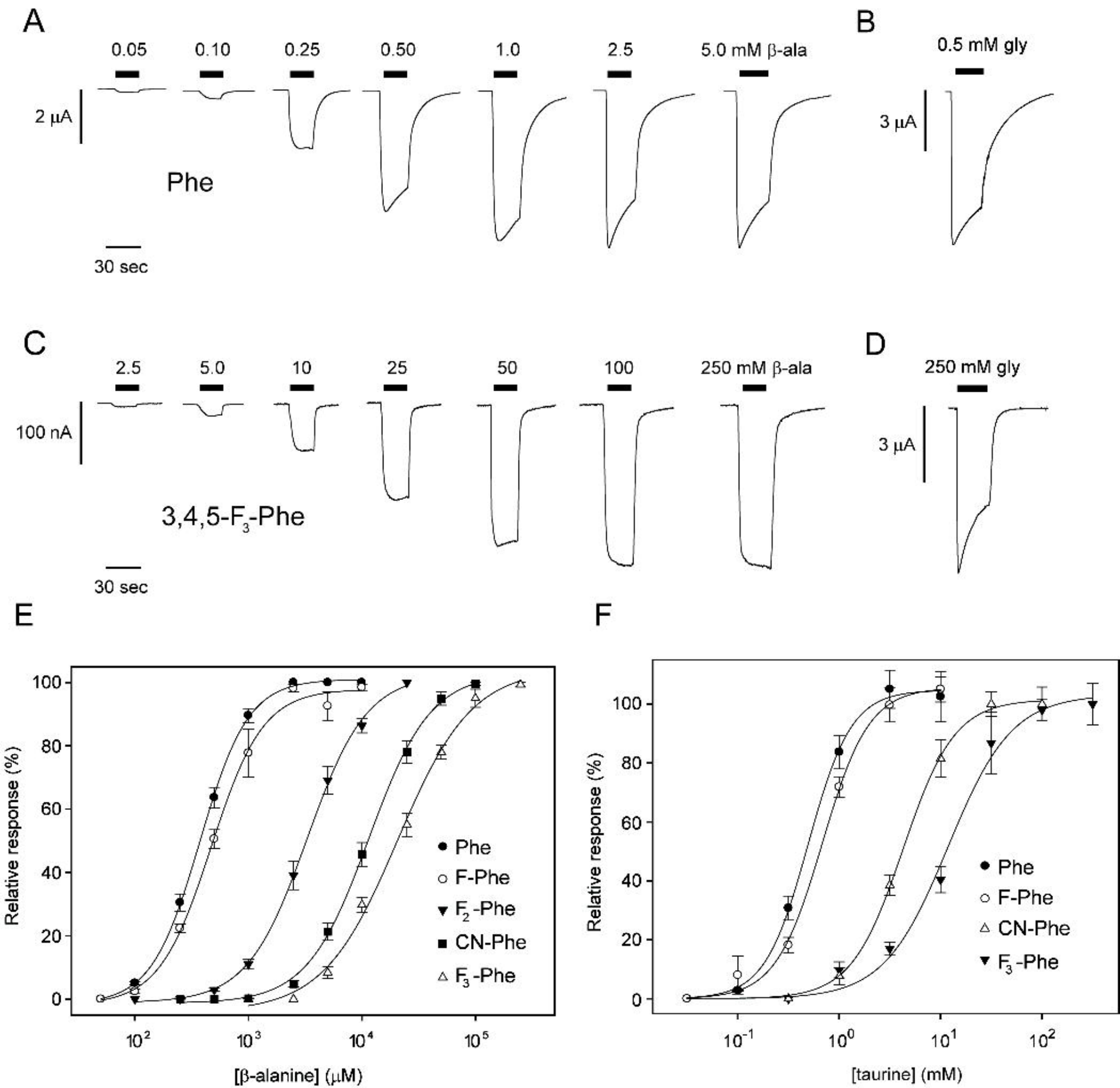

A
0.05
0.10
0.25
0.50
1.0
2.5
5.0 mM β-ala
2 μA
Phe
30 sec
B
0.5 mM gly
3 μA
C
2.5
5.0
10
25
50
100
250 mM β-ala
100 nA
3,4,5-F3-Phe
30 sec
D
250 mM gly
3 μA
E
Relative response (%)
[β-alanine] (μM)
Phe
F-Phe
F2-Phe
CN-Phe
F3-Phe
F
Relative response (%)
[taurine] (mM)
Phe
F-Phe
CN-Phe
F3-Phe

Figure 2

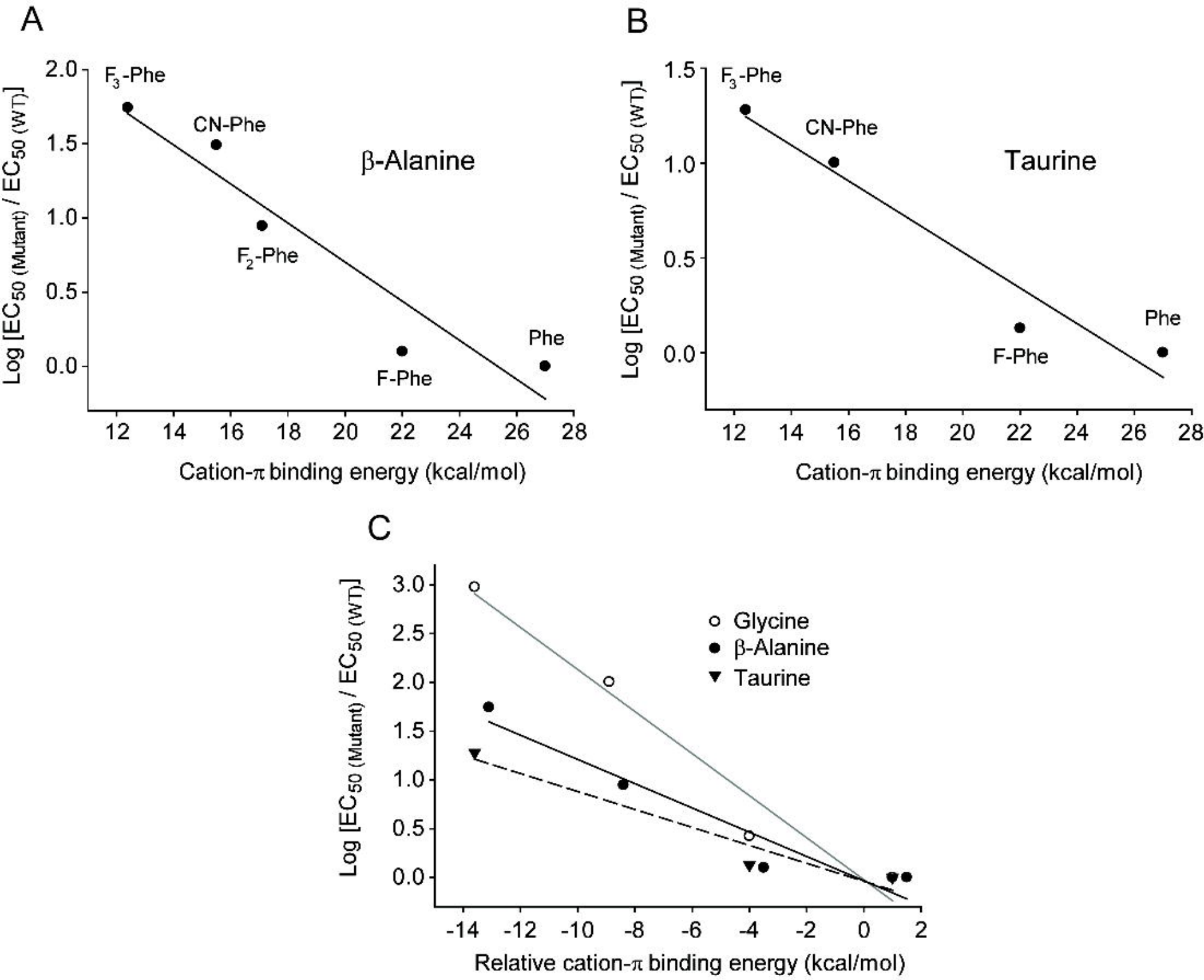
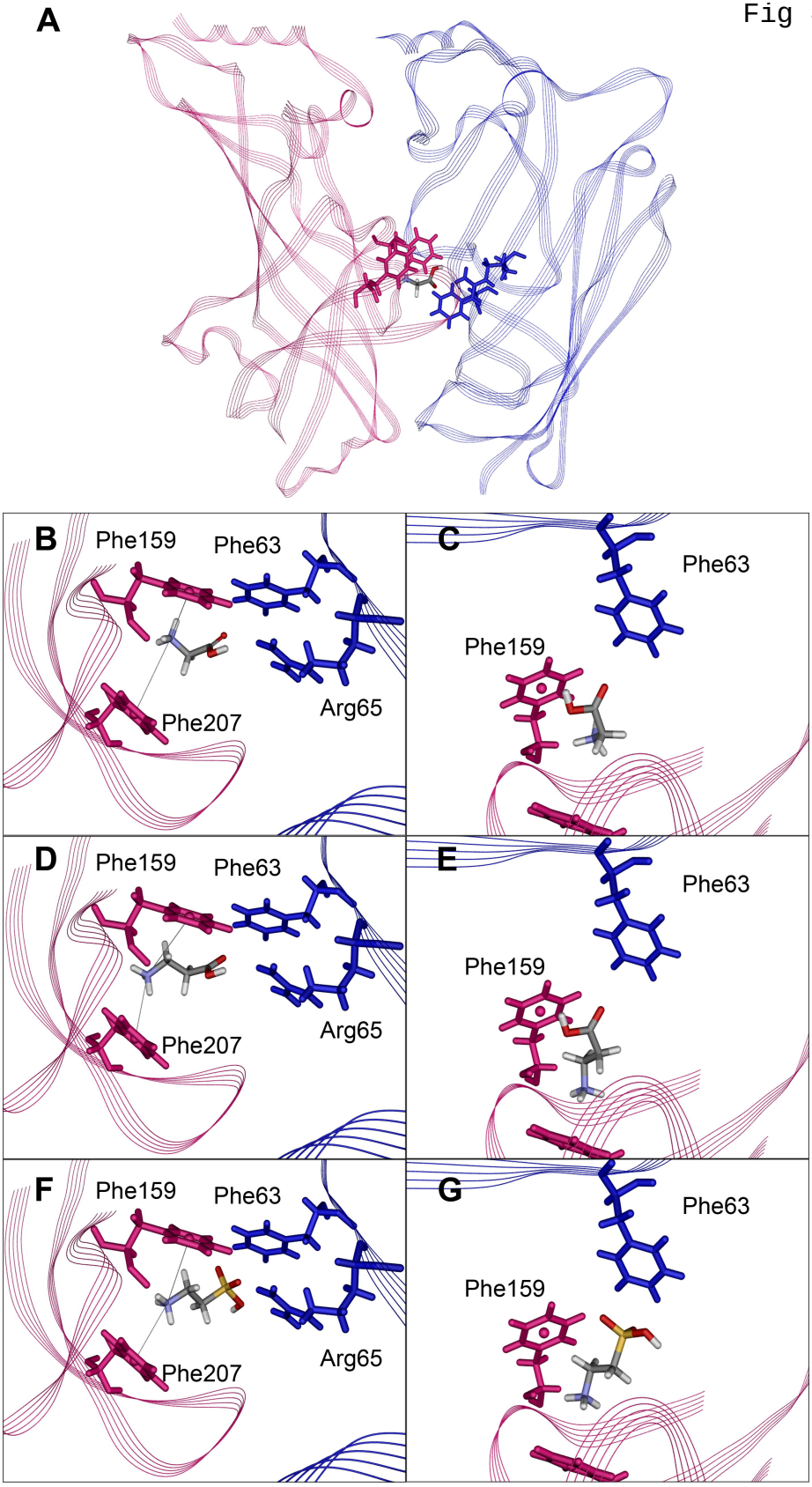

A
B
C
β-Alanine
Taurine
F3-Phe
CN-Phe
F2-Phe
F-Phe
Phe
Glycine
Log [EC50 (Mutant) / EC50 (WT)]
Cation-π binding energy (kcal/mol)
Relative cation-π binding energy (kcal/mol)

Figure 3


A
B
Phe159
Phe63
Arg65
Phe207
C
Phe63
Phe159
D
Phe159
Phe63
Arg65
Phe207
E
Phe63
Phe159
F
Phe159
Phe63
Arg65
Phe207
G
Phe63
Phe159