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A Cation-π Binding Interaction with a Tyrosine in the Binding Site of the $GABA_C$ Receptor

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Summary

$GABA_C$ (ρ) receptors are members of the Cys-loop superfamily of neurotransmitter receptors, which includes nicotinic acetylcholine (nACh), 5-HT_3, and glycine receptors. As in other members of this family, the agonist binding site of $GABA_C$ receptors is rich in aromatic amino acids, but while other receptors bind agonist through a cation-π interaction to a tryptophan, the $GABA_C$ binding site has tyrosine at the aligning positions. Incorporating a series of tyrosine derivatives at position 198 using unnatural amino acid mutagenesis reveals a clear correlation between the cation-π binding ability of the side chain and EC_{50} for receptor activation, thus demonstrating a cation-π interaction between a tyrosine side chain and a neurotransmitter. Comparisons among four homologous receptors show variations in cation-π binding energies that reflect the nature of the cationic center of the agonist.

Introduction

GABA is the major inhibitory neurotransmitter of the central nervous system, and there are three families of GABA receptors [1]. While $GABA_B$ is a G protein-coupled receptor, $GABA_A$ and $GABA_C$ receptors are homologous but distinct members of the Cys-loop superfamily of ligand-gated ion channels, which also includes nicotinic acetylcholine (nACh), 5-HT_3 (serotonin), and glycine receptors [2]. $GABA_C$ receptors are most closely related to $GABA_A$ receptors, but they have distinct kinetic and pharmacological properties, including greater sensitivity to GABA, and, unlike $GABA_A$ receptors, the ability to function as homopentamers [3–5].

As in other members of the Cys-loop superfamily, the proposed agonist binding region of the $GABA_C$ receptor is rich in aromatic residues. These are associated with several noncontiguous sequences in the extracellular domain termed loops or binding segments A–F (Figure 1A). Figure 1B shows the aromatics of binding segments B–D in four members of the superfamily. Previous studies show that in the nACh [6] and 5-HT_3 [7] receptors, a tryptophan residue on binding segment B makes a unique contribution to agonist binding through a cation-π interaction [8, 9]. In MOD-1, another serotonin-gated channel that is highly homologous to the 5-HT_3 receptor, the binding segment B residue is a tyrosine. Surprisingly, serotonin does not make a cation-π interaction with this binding segment B tyrosine in MOD-1, but instead the neurotransmitter reorients so as to make a cation-π interaction with a tryptophan on binding segment C [10]. This is consistent with the strong bias toward Trp in cation-π interactions [11].

There is, however, no tryptophan in the $GABA_C$ receptor that aligns with any predicted binding site residue. Instead, the $GABA_C$ receptor binding site is rich in tyrosines, and the residues aligned with the binding segment B or C tryptophans are both tyrosines. In the present work, we show that the B segment residue, Tyr198, contributes a cation-π interaction to agonist binding in the $GABA_C$ receptor. Given the relatively low homology of $GABA_C$ to the better characterized nAChR and 5-HT_3 receptor, this binding anchor point will be valuable in developing detailed structural models of both $GABA_C$ and the related $GABA_A$ receptors.

Results and Discussion

From our previous studies of Trp residues in other Cys-loop receptors [6, 7, 10], a straightforward strategy for evaluating a possible cation-π interaction has emerged. A fluorine substituent is deactivating in the cation-π interaction, and the effects of multiple fluorine substitutions are additive. A large, systematic decrease in agonist affinity with increasing fluorination provides compelling evidence for a cation-π interaction. The small steric size and reluctance to participate in hydrogen bonding interactions further make fluorine an ideal substituent.

Implementing this strategy for tryptophan residues was straightforward. The series Trp; 5-F-Trp; 5,7-F_2-Trp; 5,6,7-F_3-Trp; and 4,5,6,7-F_4-Trp was introduced at the site of interest, and agonist potency was measured. A comparable strategy for evaluating a tyrosine faces an additional complication. In addition to potentially making a cation-π interaction, tyrosine has the ability to participate as a hydrogen bond donor. Fluorination of tyrosine would modulate the hydrogen bonding ability of the ring. Fluorination also progressively lowers the pK_a of the tyrosine OH, such that highly fluorinated tyrosines are expected to be ionized at physiological pH [12]. Both factors would complicate any analysis of binding to a cationic ligand. Thus, it is preferable to evaluate fluorinated phenylalanine derivatives, provided that phenylalanine functions adequately in place of tyrosine at the site of interest.

As shown in Table 1, the Tyr198Phe mutant is fully functional and displays only a modest shift in EC_{50}. Thus, further modifications of the Tyr198Phe mutant

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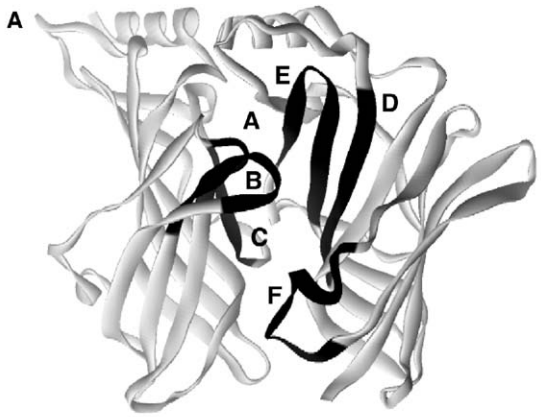

B

Receptor	B	C	C	D
nAChR	**W149**	Y190	Y198	W55
5-HT$_3$	**W183**	F226	Y234	W90
MOD-1	Y180	Y221	**W226**	F83
GABA$_C$	**Y198**	Y241	Y247	Y102

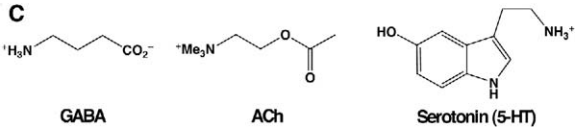

Figure 1. The Cys-Loop Superfamily of Receptors

(A) Model of the GABA$_C$ receptor extracellular domain showing two subunits. The ligand binding site lies between these subunits and is formed by residues from binding segments A–C on the principal subunit and binding segments D–F on the adjacent/complementary subunit.

(B) Amino acids that contribute to the aromatic box of the agonist binding site in four different Cys-loop receptors, including designation of the appropriate binding segment. Those residues that have a cation-π interaction with agonist are in bold.

(C) Structures of the different natural agonists that activate the receptors in (B).

can be employed to probe the agonist binding site. As such, 4-F-Phe, 3,5-F_2-Phe, and 3,4,5-F_3-Phe (Figure 2) were incorporated at position 198. Another interesting tyrosine analog is 4-MeO-Phe (or, equivalently, O-Me-Tyr), as it removes the hydrogen bond donor from the side chain of Tyr but retains the hydrogen bond acceptor and has a very similar cation-π binding ability.

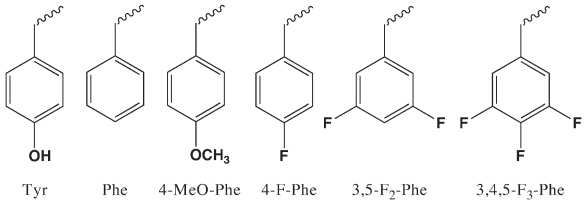

Figure 2. Structures of the Side Chains of the Natural and Unnatural Amino Acids Used in This Study

All the side chains shown in Figure 2 were incorporated into position 198 of the GABA$_C$ receptor using the in vivo nonsense suppression methodology developed previously, and the resulting mutant receptors were examined using voltage clamp analysis [13, 14]. All the receptors showed concentration-dependent GABA-induced whole-cell responses, and typical responses with F_3-Phe are shown in Figure 3. As summarized in Table 1, perturbations of the aromatic side chain at position 198 had significant and interpretable consequences. Progressive fluorination does indeed lead to large, progressive increases in EC_{50}. As in previous studies [6, 7, 10], we wished to have a semiquantitative predictor of cation-π binding ability for our modified side chains. For this, we turned to ab initio, quantum mechanical calculations of the binding of a probe cation, Na^+, to the appropriate ring. The precise identity of the cation is not critical, as it has been shown repeatedly that the *trend* in cation-π binding energies is the same regardless of the cation [8, 9]. That trend is the issue here.

The results of Table 1 are most easily appreciated with reference to the plot of Figure 4. Here we plot the cation-π binding energy versus log EC_{50} (scaled to wild-type), which transforms the experimental data to a scale that is proportional to energy. As in previous studies with Trp, an excellent linear correlation is seen across the series of Phe derivatives. The consistency of the trend and the substantial magnitude of the effect leave no doubt that the ammonium ion of GABA forms a significant cation-π interaction with the side chain of residue 198 of the GABA$_C$ receptor. We note that the functional measurement we report, EC_{50}, reflects contributions from agonist binding and gating. However, as in previous studies [6, 7, 10], we consider it safe to assume that the subtle mutations introduced here to resi-

Table 1. Substituent Effects on Cation-π Interactions and GABA$_C$ EC_{50}

Aromatic Ring	Cation-π Binding Energy (kcal/mol)[a]	EC_{50} (μM) [Hill Coeff.]
Phenol (Tyr)	26.9[b]	2.1[c] ± 0.2 [1.6 ± 0.2, n = 4]
Benzene (Phe)	27.1[b]	9.5[c] ± 1 [1.6 ± 0.1, n = 4]
Methoxybenzene	28.6	6.6 ± 2 [1.6 ± 0.2, n = 4]
Fluorobenzene	22.0[b]	34 ± 7 [1.9 ± 0.3, n = 4]
1,3-difluorobenzene	17.1	1700 ± 400 [1.4 ± 0.2, n = 4]
1,2,3-trifluorobenzene	12.9	8400 ± 1000 [1.5 ± 0.1, n = 4]

[a] 6-31G**//6-31G** calculated binding energy of Na^+ to the appropriate aromatic. Calculations were done on the simple aromatic ring, that is, benzene to model Phe, phenol to model Tyr, and so on. The EC_{50} measurements are for incorporation of the full Phe/Tyr analog.

[b] [6].

[c] Obtained by nonsense suppression.

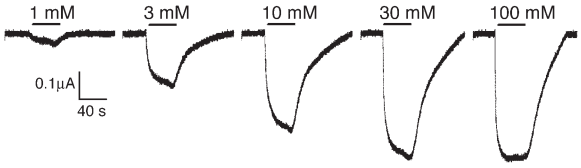

Figure 3. Exemplar of Concentration-Response Data Obtained from Two-Electrode Voltage Clamp Recordings from a Single *Xenopus* Oocyte Expressing the 198-F_3-Phe Mutant

dues that directly define that agonist binding site and presumably contact the ligand result in modulation of ligand affinity, not receptor gating.

The wild-type Tyr residue was not included in the linear fit of Figure 4. Including it does not meaningfully impact the fit, but the presentation of Figure 4 highlights an interesting observation. The EC_{50} for Tyr is ~4-fold lower than what would be expected based on the trend line for the other residues. While the effect is small, it does suggest that additional interactions to Tyr198 may be important. We propose that the enhanced affinity for tyrosine reflects the participation of the OH of Tyr198 as a hydrogen bond donor to a backbone carbonyl, a water molecule, or to GABA itself. It is well established that involving the OH of a Tyr as a hydrogen bond donor measurably enhances the cation-π binding ability of the ring [15]. However, the computed cation-π binding ability of Table 1 and Figure 4 is for the unperturbed ring. In this model, Tyr and Phe are predicted to have essentially identical cation-π binding abilities, and so the deviation from the EC_{50} trend line seen with Tyr suggests a significant hydrogen bond effect. Alternatively, the agonist could be making a favorable electrostatic interaction with the phenolic oxygen, also an effect that our small probe cation Na^+ would not sense. However, 4-MeO-Phe has a comparable oxygen substituent, and the fact that it lies on the line of Figure 4 makes this second explanation less likely.

The $GABA_C$ receptor becomes the fourth member of the Cys-loop superfamily of receptors for which a linear "fluorination plot" has revealed a cation-π interaction. For three receptors, it is an aligned residue on binding segment B: Trp149 of the nAChR; Trp183 of the 5-HT_3 receptor; and Tyr198 of the $GABA_C$ receptor. In the fourth example, MOD-1, the cation-π interaction has moved to a location on binding segment C, Trp226. Figure 5 compares these fluorination plots; MOD-1 is not shown, as its results are essentially superimposable on the 5-HT_3 line, both receptors binding serotonin to a tryptophan.

Our measure of the cation-π interaction binding ability of an aromatic system is the computed binding energy of a Na^+ cation to the ring in the gas phase. This primarily probes the electrostatic component of the cation-π interaction, because a small, focused cation like Na^+ has a very small polarizability. The full cation-π interaction does include polarization effects (such as ion-induced dipole and dipole-induced dipole interactions) and van der Waals/dispersion forces, especially with "organic" cations such as the neurotransmitters

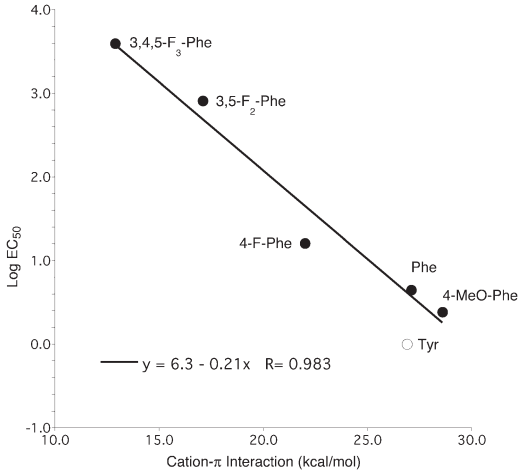

Figure 4. A Cation-π Interaction in the $GABA_C$ Receptor

Plot of the data of Table 1, and a linear fit. All EC_{50} values are scaled to wild-type. The wild-type tyrosine residue is not included in the fit. If it were, the linear fit would give: y = 6.5 – 0.225x; R = 0.974.

evaluated here. However, earlier work showed that when varying the aromatic partner in the interaction and keeping the cation constant, the trend in the cation-π interaction results exclusively from variation in the electrostatic component of the binding [16]. This justifies the fluorination plots we have used here and elsewhere. It is interesting that the quantitative impact of progressive fluorination, as judged by Na^+ binding, is essentially the same whether indole or benzene is the base aromatic. This is evidenced by the nearly perfect vertical alignment of the various data points in Figure 5. Thus, while indole (Trp) is intrinsically a stronger cation-π binder than benzene (Phe), the modulation of the electrostatic component of the cation-π interaction by fluorination is the same for the two systems. This allows a direct comparison of the three different agonists considered here: ACh, serotonin, and GABA. Intrinsically, electrostatic interactions are stronger for smaller, more focused ions [8, 9]. In the present context, that means we would anticipate that the primary ammonium (RNH_3^+) agonists serotonin and GABA should respond more strongly to variation in the electrostatic component of the cation-π interaction than the quaternary ammonium ($RN[CH_3]_3^+$) agonist ACh. Indeed, we find that the slopes of the plots in Figure 5 are steeper for serotonin and GABA than for ACh, indicating a stronger electrostatic component to the cation-π interaction. Note that a quaternary ammonium like ACh is much more polarizable than the simpler cations, and so non-electrostatic aspects of the cation-π interaction are expected to be more important for ACh.

In conclusion, our data show a strong correlation between the cation-π binding abilities of tyrosine analogs and receptor function, indicating a cation-π interaction between the primary ammonium of GABA and the side chain of tyrosine at position 198 of the $GABA_C$ receptor. These results provide further evidence of the power of the unnatural amino acid methodology to provide chemi-

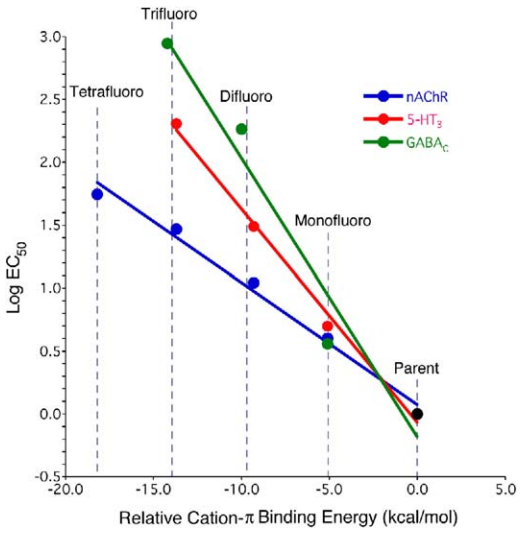

Figure 5. "Fluorination Plots" for the nACh, 5-HT_3, and $GABA_C$ Receptors

Note that the x axis has been offset, so that the parent, nonfluorinated amino acid (Trp for nACh and 5-HT_3; Phe for $GABA_C$) is given a value of zero. The actual values are 32.6 kcal/mol for Trp and 26.9 kcal/mol for Phe (Table 1). Progressive fluorination diminishes the cation-π binding ability. All EC_{50} values are ratios to the wild-type value, so all three data sets have a point at (0,0). For nACh, 5-HT_3, and $GABA_C$ receptors, respectively, the slopes (R values) are –0.097 (0.994); –0.170 (0.997); and –0.222 (0.979). The slight difference in slope for $GABA_C$ here versus Figure 4 reflects the fact that 4-MeO-Phe is not included in this fit.

cal scale information on drug-receptor interactions. This is the first demonstration, to our knowledge, that tyrosine can form such a cation-π interaction between an agonist and a neurotransmitter receptor protein. By locating the ammonium of GABA close to Tyr198, this study establishes that this amino acid is indeed in the binding pocket, providing an important anchor point for efforts to dock GABA in its binding site. As there are now an increasing number of models of the binding sites of Cys-loop receptors, an accurate knowledge of the correct orientation of the neurotransmitter is important both for understanding its mechanism of action and for drug development efforts.

Experimental Procedures

Mutagenesis and Preparation of cRNA and Oocytes

Mutant $GABA_C$ receptor subunits were developed using pcDNA3.1 (Invitrogen, Abingdon, UK) containing the complete coding sequence for the ρ1 $GABA_C$ receptor subunit kindly provided by D.S. Weiss (University of Alabama at Birmingham). The tyrosine codon at position 198 was replaced by TAG as previously described [7]. Mutagenesis reactions were performed using the Kunkel method, and confirmed by DNA sequencing [17]. Wild-type and mutant receptor subunit coding sequences were then subcloned into pGEMHE [18]. This was linearized with Nhe1 (New England Biolabs, Ipswich, MA) and cRNA was synthesized using the T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX). Oocytes from *Xenopus laevis* were prepared and maintained as described previously [14].

Synthesis of tRNA- and dCA-Amino Acids

Unnatural amino acids were chemically synthesized as nitroveratryloxycarbonyl (NVOC)-protected cyanomethyl esters and coupled to the dinucleotide dCA, which was then enzymatically ligated to 74-mer THG73 $tRNA_{CUA}$ as detailed previously [14]. Immediately prior to coinjection with mRNA, tRNA-amino acids were deprotected by photolysis. Typically 5 ng mRNA and 25 ng tRNA-amino acids were injected into Stage V-VI oocytes in a total volume of 50 nl. For control experiments, mRNA was injected (1) in the absence of tRNA and (2) with the THG73 74-mer tRNA. Experiments were performed 24–72 hr postinjection.

Characterization of Mutant Receptors

GABA-induced currents were recorded from individual oocytes using an OpusXpress system (Molecular Devices Axon Instruments, Union City, CA) as previously described [19]. Briefly, experiments were performed at 22–25°C. GABA (Sigma, St. Louis, MO) was freshly prepared daily, diluted in ND96, and delivered to cells via a computer-controlled perfusion system. Glass microelectrodes were backfilled with 3 M KCl and had a resistance of approximately 1 MΩ. The holding potential was –60 mV. EC_{50} values were determined from dose-response data using PRISM software (GraphPad, San Diego, CA).

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