HHS Public Access

Author manuscript

*J Mol Biol*. Author manuscript; available in PMC 2021 August 20.

Published in final edited form as:

*J Mol Biol*. 2021 August 20; 433(17): 167035. doi:10.1016/j.jmb.2021.167035.

# Cation-π interactions and their functional roles in membrane proteins

**Daniel T Infield**[1], **Ali Rasouli**[2,3], **Grace D Galles**[1,4], **Christophe Chipot**[2,5,6], **Emad Tajkhorshid**[2,3], **Christopher A Ahern**[1]

[1]Department of Molecular Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52246, U. S. A.

[2]Theoretical and Computational Biophysics Group, NIH Center for Macromolecular Modeling and Bioinformatics, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U. S. A.

[3]Department of Biochemistry, and Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U. S. A.

[4]Unnatural Protein Facility, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, U. S. A.

[5]Laboratoire International Associé CNRS-University of Illinois, Unité mixte de recherche n°7019, Université de Lorraine, France

[6]Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U. S. A.

## Abstract

Cation-π interactions arise as a result of strong attractive forces between positively charged entities and the π-electron cloud of aromatic groups. The physicochemical characteristics of cation-π interactions are particularly well-suited to the dual hydrophobic/hydrophilic environment of membrane proteins. As high-resolution structural data of membrane proteins bring molecular features into increasingly sharper view, cation-π interactions are gaining traction as essential contributors to membrane protein chemistry, function, and pharmacology. Here we review the physicochemical properties of cation-π interactions and present several prominent examples which demonstrate significant roles for this specialized biological chemistry.

## Graphical Abstract

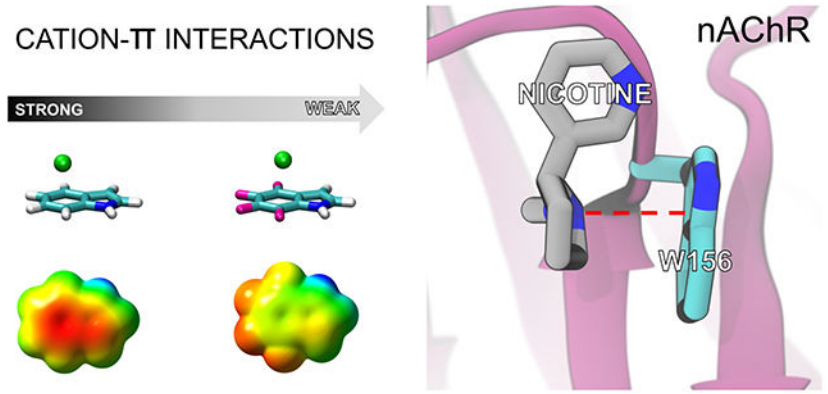
CREDIT Author statement

All authors contributed to Writing- Original draft preparation, Visualization, Investigation. Writing- Reviewing and Editing,



Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.




## Introduction

### Physicochemical properties

Cation-π interactions are short-range, noncovalent interactions between cations and nearby π systems (Figure 1). In the context of membrane biology, these energetically significant interactions have multiple advantageous characteristics which have been highlighted in studies employing gas and organic-phase "reduced systems" in combination with *ab initio* computations. For instance, whereas the strength of a salt bridge formed between acidic carboxylates (Glu, Asp) and basic moieties (Arg, Lys) is primarily determined by the distance separating the two charge centers (r), cation-π interactions have an additional geometric "en face" prerequisite that the cation engages directly with the face of the aromatic [1]. This coordination geometry allows the cation to directly interact with the quadrupole moment of the aromatic ring. The polarization-driven, attractive forces in cation-π interactions stem from two distinct terms. The first term arises from the electric field generated by the cation, which polarizes the π-electron cloud of the aromatic side chain. The second term relates to the induced dipole moment of the aromatic ring, which interacts with the polarizing positive charge via its electrostatic potential. Put together, the attractive part of the short-range potential that accounts for induction effects in cation-π interactions can formally be described analytically by means of a $1/r^4$ term, a term that has been added to non-polarizable force fields [2–4] to approximate cation-π interactions in classical simulations. For the interested reader, details relating to the computational efforts to formally include induction phenomena via explicit integration [5–9], and/or by the development of other models that remain within the framework of the pairwise additive approximation [10, 11] are more extensively described elsewhere [9, 12, 13].

In biology, consenting cations can be found in the basic side chains of proteins, as well as many different ligands, toxins, other small molecules, or even ions that might closely interact with the protein (Figure 1). Similarly, the π-electron partner in a cation-π interaction can be provided either by aromatic side chains (Phe, Tyr, or Trp), or by an aromatic moiety of an interacting ligand. Note that the side chain of histidine may formally act as a cation or as an aromatic group, thus requiring particular consideration [14]. For the guanidinium moiety of arginine–a dispersed π-system itself–the side chain can interact with an aromatic through parallel (stacking) or perpendicular (T-shaped) geometries (Figure 1B–

C). Important in the context of membrane proteins, which are exposed to mixed lipid and aqueous environments, cation-π interactions are often found at the surface of proteins [1]. This suggests their higher resilience to the screening effects of solvent than salt bridges, which are more commonly found interred, or at the interface between two proteins or domains [1]. While a salt bridge between Asp/Glu and Arg/Lys residues can reach nearly the strength of a covalent bond in a low-dielectric medium (~60kcal/mol), this interaction strength rapidly dissipates with solvation [1, 15]. In contrast, a cation-π interaction, which is weaker than a salt bridge in a low-dielectric environment (~20kcal/mol), is less drastically affected by solvation [1, 12], such that a cation-π interaction is estimated to be 2.5- to 10-fold stronger than a comparable salt bridge in water [1, 12, 15]. As proposed by Gallivan and Dougherty, the resilience of cation-π interactions in an aqueous environment relative to salt bridges is explained at least in part by the fact that for a salt bridge to form in water, both sides of the interaction must pay an energetic desolvation penalty, while in a cation-π interaction only the cation suffers this penalty [1]. The mechanistic implications of both the environmental resilience and the geometric dependence of cation-π interactions can be profound for the diverse biological environments that membrane-embedded proteins experience during their function.

## A means to quantify cation-π interactions in biology

The biological role of a putative cation-π interaction is not easily quantified experimentally. Side-chain removal of a benzene (Phe), phenol (Tyr), or indole (Trp) moiety through conventional mutagenesis will fully remove the π-electron contribution as well as most of the steric and hydrophobic properties of the aromatic. Should a particular phenotype persist after substitution with a non-aromatic amino acid (yet still hydrophobic, e.g., Leu), then the functional requirement of a cation-π interaction can be ruled out.[1] However, no such "natural" substitution enables a strategy for energetic estimation or validation of the cation-π interaction at play.

For this purpose, serial fluorination of the aromatic side chain has become the go-to method for the experimental study of cation-π interactions and their importance in maintaining function. The electron-withdrawing fluorine substitution is nearly isosteric to hydrogen and does not alter aromatic planarity (FIGURE 2A). Importantly, successive fluoro-substitution withdraws electron density from the aromatic face in a linear fashion [16]. Thus, each fluorine atom added will result in an approximately equal drop in the cation-π binding energy, pursuant to dissipation of the electrostatic component of the cation-π interaction. Note that as alluded to above, the cation-π interaction undoubtedly involves additional types of intermolecular forces beyond electrostatics. This being said, electrostatics have been established to be highly predictive of the overall strength of the cation-π interaction [17]. Experimentally, a loss of cation- π interaction energy could be reflected in binding affinity or efficacy (in the case of ligands) or shifts in biophysical properties such as gating energetics for ion channels. For a *bona fide* cation-π interacting pair, plotting the

[1]We use this specific language ("functional requirement") because in biological contexts, the formal possibility often exists that elimination or alteration of an aromatic group may be functionally silent due to compensation. For this reason, it is more straightforward to confirm a cation-π interaction than to conclusively refute one, particularly when testing one aromatic within a network.

experimental binding energy for each fluoro-aromatic condition against a calculated binding energy of an ideal system ($Na^+$-bound benzene for instance), will reveal a linear relationship [16].

Despite the power of the methodology in dissecting the importance of cation-π interactions, the site-specific encoding of fluoro-aromatic amino acids is a non-trivial undertaking which requires genetic code expansion methodologies. For proteins that tolerate prokaryotic expression, autotrophic variants have been developed for encoding of some fluoro-aromatics in response to natural codons [18]. However, more complicated chemical-biology approaches are required for the site-specific encoding of fluoro-aromatics, particularly within eukaryotic membrane proteins.

To encode unnatural aromatic variants in a eukaryotic cell, methods first developed in the Schultz lab [19] and further honed by Dougherty and Lester [20] have produced a general protocol, termed *in vivo* nonsense suppression, to encode a variety of unnatural amino acids. Briefly, the method utilizes an amber (TAG) stop-codon-suppressing orthogonal tRNA, which is enzymatically appended at the 3' end with an unnatural amino acid of interest e.g., a fluoro-aromatic. The property of orthogonality refers to the competency of the acylated tRNA for ribosomal translation, as well as its incompatibility with endogenous amino acid synthetases (FIGURE 2B). Thus, the ideal orthogonal tRNA is not significantly edited or reacylated, thereby reducing the potential for spurious encoding of natural amino acids [21, 22]. The acylated orthogonal tRNA is co-injected into the *Xenopus laevis* oocyte expression system with an RNA encoding the protein of interest with an inserted amber (TAG) stop codon at the desired encoding site (FIGURE 1B). The oocyte is useful for this purpose as it is a large (~1μl), easy to inject, nondividing cell with relatively low endogenous ion channel activity. It is suitable for high-resolution electrophysiological approaches commonly used to characterize ion channel and transporter activities. Nonsense suppression with *in vitro* chemically acylated tRNA is inherently inefficient in the production of full-length proteins, stemming both from the amber codon suppression itself and the non-renewable nature of the acylated tRNA. In this regard, the study of ion channels is especially suited to the approach given that a single channel can conduct millions of ions per second, which can be easily recorded as electrical current. Indeed, a so-called macroscopic recording of ion channel behavior in an oocyte is possible from the plasma-membrane expression of only a subfemtomole quantity of channels on the oocyte surface.

## Examples of cation-π interactions in membrane proteins

Biological examples of cation-π interactions may be thought to fall under two broad categories. The first are those found *within* or *between* proteins, involving basic and aromatic amino acids, while the second encompasses soluble ligands and receptors. The former proteinaceous interactions may be important for stabilizing overall structure or particular conformational states, and were observed to be abundant in proteins of known structure back when the PDB structural database was dominated by soluble proteins [23]. Whether such structural cation-π interactions are similarly common in membrane proteins is less well understood. This is due to a historical paucity of unique membrane protein

structures, which has begun to change with the advancement of protein engineering methods [24], as well as single particle cryo-EM [25].

To estimate the abundance and nature of cation-π interactions within membrane proteins, we provide a survey of proximity between aromatic (Phe/Tyr/Trp) and basic (Arg/Lys) residues for ion channels and G protein-coupled receptors (GPCRs) of known structure (203 and 99 unique proteins, respectively; https://blanco.biomol.uci.edu/mpstruc/). These structures arise from data of variable resolution and quality; thus, prudence should be used in the assignment of likely cation-π interactions. A range of distance thresholds have been proposed for energetically significant cation-π interactions, from less than 3.7 Å [11] to 6 Å, depending on the molecules involved [16]. The angle of approach, $\theta$, with respect to the normal of the aromatic ring is also an important factor, as interactions become unfavorable beyond 45° [11]. Distance and relevant angles of approach for all interactions within 8 Å are plotted in FIGURE 3. For lysine, amino acid pairs most likely to be true cation-π interactions are represented by tall bars located on the near left side of the 3D plot, which feature the smallest separations and deviations from an ideal angle of approach (FIGURE 3A and C). In the case of the interaction of arginine with aromatic amino acids, most highly favored stacked interactions are clustered near where (in this case, two) angles of approach ($\theta_1$, $\theta_2$) are close to 0° (FIGURE 3B and D). In those regions, there is an apparent preponderance of ideal arrangements of the guanidinium moiety with π-electron clouds, and this correlates with close proximity (approximately 3.5-Å distance). As previously discussed, Arg can also form cation-π interactions through an alternate, T-shaped organization. In this plot, a T-shaped organization would involve angles approaching 0° for $\theta_1$, and 90° for $\theta_2$, respectively. This geometry corresponds to a slightly greater separation, i.e., approximately 4.0 Å. In terms of simple distance, statistics for these data give an overall impression of higher preponderance of likely cation-π interactions involving arginine (118, 714, and 1781 occurring within 4, 5, or 6 Å, respectively) compared to lysine (58, 386, and 949 occurring within 4, 5, 6 Å, respectively); thus mirroring the proportions previously found in soluble proteins [26].

A second category of biological cation-π interactions embraces ligand-receptor interactions. In this category, a “ligand” may be defined in a broad sense to include neurotransmitters [27–29], drugs [30–32] , simple cations such as metals [33, 34], toxins [35–37], and even potentially the choline headgroups of some membrane lipids [38, 39]. Below we present a targeted survey of several instances of cation-π interactions from multiple protein families. The interactions we detail below feature aromatic amino acids and cationic ligands. Using the method of nonsense suppression with chemically misacylated tRNA, more than 30 such cation-π interactions have been identified and quantified between surface-exposed aromatic amino acids in receptors and soluble cations (often basic amine-bearing groups). We note also, however, that cation-π interactions involving basic (e.g. Arg, Lys) amino acids and the aromatic groups on ligands have been demonstrated. One particularly interesting example involves the K2P channels TREK1 and TREK2 and their binding of small molecule activators ML335 and ML402 [32]. In this instance, a resident Lys on the channel interacts with an aromatic group on the drug via a cation-π interaction whose role in ligand binding was elegantly confirmed via synthesis and testing of an analog of ML335 lacking

aromaticity in the interacting ring. Below, when appropriate, we combine structural and functional data to inform our analyses of the interactions.

## Pentameric ligand-gated ion channels- focus on nAChR

The nicotinic acetylcholine receptor (nAChR) is the founding member of a family of eukaryotic pentameric ligand gated ion channels, which also includes receptors for glycine, serotonin, GABA, and glutamate. Acetylcholine (ACh) was recognized early on as a potential cation-π forming ligand and was tested for this possibility against a purely synthetic aromatic receptor [23, 40–42]. Soon thereafter, the structure of the acetylcholine esterase demonstrated the biological relevance of a cation-π interaction between ACh and a resident tryptophan in the enzyme [43]. Encouragingly, contemporary research established that the binding site of ACh on nAChR was rich in aromatic residues [44]. Using their recently developed chemical misacylation approach for nonsense suppression described above, the Lester and Dougherty research groups systematically encoded serially fluorinated tryptophan analogs at the implicated sites and ultimately found a strong cation-π interaction involving a single tryptophan residue, W149, of the α subunit [29].

These findings were supported by structures of the ACh-binding protein, a homolog of the ligand-binding domain of nAChR [45]. Atomic structural determination of the full, heteromeric nAChR would not be accomplished for another 15 years (FIGURE 4, center). As expected, the structure of the human α4β2 heteropentamer bound to nicotine showed the ligand enveloped in an aromatic cage located at the interface of α4 and β2 subunits, with W156 of α4 (analogous to W149 discussed above) positioned to interact with the amine group of nicotine [46] (FIGURE 4, top left). The pose of the drug in the binding pocket helps explain why Lester and Dougherty did not find cation-π interactions between nicotine and other nearby tryptophan residues such as W57 of β2, which interacts with nicotine, albeit not in an orientation ideal for cation-π interaction.

Apart from this high-resolution map of the nicotine binding site within the α4/β2 interface, the structure of the channel also revealed an interesting motif at a corresponding region in non-ligand binding interfaces, such as β2/β2. While this interface lacks the features to support ligand binding, it contains instead a structural (proteinaceous) cation-π interaction consisting of a subunit-specific arginine sandwiched between two tyrosine residues (FIGURE 4, top right). Recently, the native *Torpedo* nAChR structure bound to the inhibitor bungarotoxin was solved via cryo-EM and the interaction of these entities in the α4/β2 ligand binding site represents a sort of conceptual merge of the motifs described above [36]. Two amino acids from bungarotoxin contribute an arginine (R36), as well as a phenylalanine (F32) to a cation-π sandwich involving Y198 from the α subunit of nAChR (FIGURE 4, bottom left) [36]. Thus, this deadly neurotoxin blocks the binding of the model cation-π ligand ACh to its receptor by substituting a proteinaceous cation-π interaction of its own.

Beyond the nAChR, it bears mention that energetically significant cation-π interactions have been identified between ligands and aromatic amino acids located within orthosteric binding sites in other pentameric ligand-gated ion channels, including GABA [47, 48], glycine [27], and serotonin [28] receptors. The overall molecular logic is conserved, though in some cases the binding sites utilize different aromatic residues.

## Voltage gated ion channels- focus on $Na^+$ channel pharmacology

Voltage-gated $Na^+$ channels ($Na_V$s) promote rapid depolarization during the action potentials of the excitable cells of nerve and muscle [49]. These large (~250kD) 24 transmembrane (TM) proteins have four domains (DI-DIV), each with six helical TM segments, termed S1–S6. The S1–S4 segments of each domain are assigned as peripheral "voltage-sensing" domains, and the S5–S6 TM segments, connected by a reentrant loop, define a central $Na^+$-selective pore. The selectivity filter is located at an extracellular-facing constriction, which further expands to an intracellular, water-filled vestibule lined by the four S6 segments from each domain.

Inherited mutations or post-translational dysregulation of $Na_V$s can result in aberrant channel activity and pathophysiological $Na^+$ conductance. In some cases this can entail destabilization of non-conducting "deactivated" or "inactivated" channel states. Such 'hyper-excitable' $Na_V$ channel gating can result in cardiac arrythmia [50], epilepsy [51], and enhanced pain syndromes [52]. For this reason, therapeutics that inhibit $Na_V$ channel activity are widely used in clinical settings for the treatment of these disorders. Several local anesthetics as well as anti-arrhythmic and anti-epileptic agents are weakly basic, amine-bearing $Na_V$ channel blockers. To bind the channel, these drugs rely upon a pair of conserved aromatic groups found in the DIV S6 pore-lining helix [53–55]. Removal of either aromatic residue results in decreased channel block [53–55]. Using site-directed serial fluorination of either aromatic site, it has been shown that the aromatic proximal to the selectivity filter engages lidocaine and other class 1b anti-arrhythmic drugs via cation-$\pi$ binding, while the lower aromatic does not [30, 31]. Interestingly, other anti-arrhythmic drugs designated as class 1a or 1c lack a cation-$\pi$ component at either aromatic, suggesting that multiple binding modes may exist within the $Na_V$ channel inner vestibule [30].

Consistent with their role in action potential firing of nerve and muscle tissues, $Na_V$ channels are also targeted by toxins used in predator-prey relationships [56]. Tetrodotoxin and saxitoxin (TTX and STX), for instance, are cationic neuromuscular toxins that rapidly and reversibly block $Na_V$ channels via an aqueous extracellular binding site [57]. Nine mammalian $Na_V$ channel isoforms are commonly segregated as either sensitive ($Na_V$1.1-$Na_V$1.4, $Na_V$1.6, $Na_V$.1.7) or resistant ($Na_V$1.5, $Na_V$1.8, $Na_V$1.9) to TTX, showing blocking activity in nanomolar concentrations or micromolar concentrations of the toxin respectively. Those in the higher-affinity category have a conserved aromatic (Tyr or Phe) located just extracellular to the selectivity filter ring in the DI S5–S6 loop, whereas those that bind poorly lack the aromatic side chain, and instead have a Cys or Ser at this site [57]. Mutagenesis to swap a non-aromatic for a Phe or Tyr significantly enhances TTX binding affinity, suggesting a requirement for an aromatic residue at this conserved position [58]. Consistent with this hypothesis, it has been shown by encoding fluoro-aromatics in the skeletal $Na_v$ 1.4 isoform that the guanidinium group of the TTX neurotoxin binds the channel via a cation-$\pi$ interaction [35]. Interestingly, the inhibition of $Na_v$ channels by $Ca^{2+}$ has been shown to rely on a cation-$\pi$ interaction with the same aromatic [33].

Recent near-atomic cryo-EM structures from the human neuronal channel h$Na_V$ 1.7 bound to TTX or STX revealed essentially identical binding sites within the channel pore, consistent with prior functional studies and featuring extensive interactions with the toxin

[59]. Here, we focus on the region proposed to include a cation-π interaction with STX (FIGURE 5A). Specifically, three critical residues are positioned to interact with the guanidinium groups of STX; a conserved tyrosine (Y362) and two acidic residues (E364 and E930) located on either side of it. Mutation of any of these three residues significantly reduces affinity for the toxin [60]. From this structure it appears that the aromatic residue (Y362) and E930 engage the six membered guanidinium-bearing ring of the toxin, while E364 engages the neighboring five-membered ring.

As an interesting aside, it is known that several species of frogs express and secrete a protein called saxiphilin, which binds STX with high affinity and is thought to confer resistance to the toxin [61]. Recently, a high-resolution crystal structure of the saxiphilin:STX complex was solved, allowing for assignment of molecular interactions that comprise the STX binding site [37]. Saxiphilin uses a strikingly similar chemistry to engage STX as do $Na_V$ channels. This includes a motif consisting of two acidic residues flanking an aromatic residue, in this case a Phe (FIGURE 5B). The saxiphilin:STX structure suggests that for this complex, the aromatic is engaged in a cation-π interaction with the five-membered guanidinium-bearing ring of STX, and that this interaction is supported by the two acidic residues. Saxiphilin is not evolutionarily related to $Na_V$ channels, and the three amino acids comprising this electrostatic recognition motif are contained within a single short loop, rather than across multiple domains. With this in mind, it is remarkable that this protein evolved such a similar recognition strategy to that of the ion channel. One might even say that nature has strongly “suggested” a binding strategy to be used in future efforts for therapeutic neutralization of STX.

## G protein-coupled receptors (GPCRs)- focus on Muscarinic Acetylcholine Receptor

GPCRs serve to couple external chemical signals to intracellular signaling pathways through the activation of their cytoplasmic partners, G-proteins. Approximately one third of all FDA approved drugs act on GPCRs [62]; thus, the atomic-level understanding of their mechanisms of action is of critical clinical importance. The chemical mechanisms of molecular recognition by GPCRs reflect the diversity of the proteins (at least five major classes comprising ~800 individual proteins [62]) and the ligands involved.

In general, regulation of GPCRs is accomplished by the binding of orthosteric as well as allosteric ligands to distinct sites [63]. For the prototypical class-A M2 muscarinic receptor (M2R), structural data support the idea that cation-π interactions play significant roles in the recognition of common cationic functional groups within ligands by specific aromatic residues within the protein. For example, binding of the drug Iperoxo has been proposed to involve cation-π interactions between its trimethyl ammonium group and a box of aromatic (tyrosine) residues lining the orthosteric ligand binding site in the receptor (FIGURE 6) [64]. Binding is also supported by a nearby Asp residue. It is worth noting that the trimethyl ammonium group in Iperoxo is shared with ACh, the physiological agonist of the receptor, suggesting that recognition of the endogenous ligand also depends on cation-π interactions.

It has been similarly proposed that binding of at least a subset of allosteric modulators of M2R relies on cation-π interactions. A study combining computational docking and simulation identified a common binding site for a gamut of allosteric modulators that is

immediately extracellular to the orthosteric binding site and features a different set of aromatic groups [65]. The docking results suggested that these aromatics may interact with cationic centers on the drug via cation-π interactions. *In vitro* experimentation using conventional mutagenesis supported the idea that binding of the prototypical modulator $C_7$/3-phth is dependent on such cation-π interactions. General support for the identification of this region as an allosteric binding site on M2R comes from a crystal structure of the protein solved with the allosteric modulator LY2119620 bound [64]. LY2119620 is structurally distinct from $C_7$/3-phth. However, it binds to an overlapping site, utilizing its own aromatic groups to interact with the aromatics on M2R. The structural diversity among the allosteric modulators guarantees that their binding to similar sites is mediated by a range of different chemistries.

In addition to the M2R, another example of a cation-π interaction involved in GPCR function is found in the binding of glutamate to metabotropic glutamate receptors. In mGluR1, binding involves interactions between the α amino group of glutamate and a conserved tyrosine residue (Y236) in the extracellular ligand binding domain on the receptor [66, 67]. As with multiple previously mentioned cation-π interactions ($Na_v$-STX, Saxiphilin-STX, and M2R-iperoxo), this motif is supported by an ionic interaction with a nearby acidic residue (D318) to the same cationic group. Conventional mutation of both residues affects ligand binding [68]. Finally, in addition to the interactions discussed above, we note that there is great potential for the discovery of novel cation-π interactions in future GPCR research since there is currently very little known about the molecular basis of recognition for many GPCR ligands such as odorants [69].

## Integrins

Cation-π interactions have been proposed to be critical to the structure and function of the membrane-associated integrin complex in platelets. Specifically, it has been proposed that a conserved aromatic (Phe or Tyr) residue in the βI subunit interacts with divalent metal ions via a cation-π interaction, and that this interaction is crucial for ligand binding and strong $Mg^{2+}$-dependent cell adhesion [34]. This interaction is supported by crystal structures that locate a row of cations in the proximity of this conserved aromatic residue, with an orientation suggestive of a cation-π interaction [70].

Experimental support of this interaction has thus far been provided by reconstitution of the platelet adhesion mechanism of the α4β7 complex in transfected HEK 293T cells [34]. Conventional point mutation of F185 supports the potential of an energetically significant cation-π interaction at this site, in as much as tyrosine is interchangeable at this position, whereas non-aromatics such as alanine, leucine, and glutamate are not. Intermediate phenotypes were observed for the aromatic residues tryptophan and histidine; this observation was reasoned to stem from improper fit of these differently-shaped amino acids in the interaction site. FRET analysis of the point mutants supported the idea that this cation-π interaction helps maintain the proper orientation of the extracellular domain relative to the plasma membrane [34].

## Conclusions

Cation-π interactions are critical to the function and pharmacology of several types of ion channels and other membrane proteins. These interactions result from the attraction of the charge borne by a positive center towards the quadrupole created by the π-electron cloud of an aromatic side chain. Compared to salt bridges, cation-π interactions are relatively more resilient to water and have an added geometric component that strongly influences energetics. Here, we focused on cation-π interactions in specific membrane proteins that have been studied by various methodologies. There is reason to believe that many new and interesting cation-π interactions will be proposed on the basis of continually emerging atomic and near-atomic membrane protein structures solved via X-ray crystallography and single-particle cryo-EM. These structures have in fact changed the way we think about "testing" or "confirming" the structural and functional roles of cation-π interactions in membrane proteins. Previously, in the absence of sufficient high-resolution structural data, a functionally demonstrated cation-π interaction provided incredibly unique high-resolution information about the interaction between two amino acids, or between an amino acid and a ligand, which may not have been otherwise knowable. The subsequent emergence of structural information would in many cases support the prior inference. The solving of representative members of many ion channel and other membrane protein families has created a scenario wherein structural data can more often serve as a starting point to propose cation-π interactions. These interactions may then be tested for energetic significance, and, therefore, a functional role, via complementary approaches including the encoding of fluorinated amino acids via nonsense suppression.

In what new contexts can we forge this ultimate link between how an interaction *looks* and how it *works*? To this end, significant effort must be expended towards the development of new methodological platforms for the encoding of unnatural amino acids outside of the *Xenopus* oocyte. In principle, several routes could be explored. Simply translating the chemical misacylation method to mammalian cells via optimized and/or novel delivery techniques is an option that may increase the applicability of the approach to protein targets requiring mammalian expression hosts [71]. However, inherent limitations in these technologies with efficiency and scaling make their application to biochemical assays difficult if not impossible. Alternatively, *in vivo* nonsense suppression via co-evolved tRNA / synthetase pairs offers facile, scalable encoding of unnatural amino acids in mammalian cells in instances where, *crucially*, an efficient and high-fidelity synthetase has been successfully identified and validated for the amino acid(s) of interest. Feasibility is suggested by a study reporting a synthetase that is competent to encode a select number of fluorinated phenylalanine residues via nonsense suppression in *E. coli*.[72]. The emergence of such tools would be expected to significantly grow and democratize the study of cation-π interactions, allowing a deeper understanding of their significance in biology.

## Acknowledgements

We thank Colin Clark and Frederico Sassoli Fazan for helpful comments with the manuscript.

Funding provided by the Agence National de la Recherche (ProteaseInAction) to C.C NIH/NHBLI for NIH F32HL149184 to D.T.I., NIH P41-GM104601 to ET, NIH/NINDS R24-NS104617 to C.A.A

## References


1. Gallivan JP, Dougherty DA, A Computational Study of Cation-π Interactions vs Salt Bridges in Aqueous Media: Implications for Protein Engineering. Journal of the American Chemical Society, 2000. 122(5): p. 870–874.
2. Turupcu A, Tirado-Rives J, and Jorgensen WL, Explicit Representation of Cation-pi Interactions in Force Fields with 1/r(4) Nonbonded Terms. J Chem Theory Comput, 2020. 16(11): p. 7184–7194. [PubMed: 33048555]
3. Li P, Song LF, and Merz KM Jr., Parameterization of highly charged metal ions using the 12-6-4 LJ-type nonbonded model in explicit water. J Phys Chem B, 2015. 119(3): p. 883–95. [PubMed: 25145273]
4. Minoux HC, Chipot C , Cation–π Interactions in Proteins: Can Simple Models Provide an Accurate Description? Journal of the American Chemical Society, 1999. 121: p. 10366–10372.
5. Caldwell JW and Kollman PA, Cation-Pi Interactions - Nonadditive Effects Are Critical in Their Accurate Representation. Journal of the American Chemical Society, 1995. 117(14): p. 4177–4178.
6. Archambault F, et al., Polarizable intermolecular potentials for water and benzene interacting with halide and metal ions. J Chem Theory Comput, 2009. 5(11): p. 3022–3031 [PubMed: 21113276]
7. Lamoureux G, MacKerell AD, and Roux B, A simple polarizable model of water based on classical Drude oscillators. Journal of Chemical Physics, 2003. 119(10): p. 5185–5197.
8. Orabi EA and Lamoureux G, Cation-pi Interactions between Quaternary Ammonium Ions and Amino Acid Aromatic Groups in Aqueous Solution. J Phys Chem B, 2018. 122(8): p. 2251–2260. [PubMed: 29397727]
9. Rupakheti CR, et al., Modeling induction phenomena in amino acid cation-pi interactions. Theoretical Chemistry Accounts, 2018. 137(12).
10. Chipot C, et al., Molecular dynamics potential of mean force calculations: A study of the toluene-ammonium pi-cation interactions. Journal of the American Chemical Society, 1996. 118(12): p. 2998–3005.
11. Levitt M and Perutz MF, Aromatic rings act as hydrogen bond acceptors. J Mol Biol, 1988. 201(4): p. 751–4. [PubMed: 3172202]
12. Liu H, et al., Accurate Description of Cation-pi Interactions in Proteins with a Nonpolarizable Force Field at No Additional Cost. J Chem Theory Comput, 2020. 16(10): p. 6397–6407. [PubMed: 32852943]
13. Khan HM, MacKerell AD Jr., and Reuter N, Cation-pi Interactions between Methylated Ammonium Groups and Tryptophan in the CHARMM36 Additive Force Field. J Chem Theory Comput, 2019. 15(1): p. 7–12. [PubMed: 30562013]
14. Liao SM, et al., The multiple roles of histidine in protein interactions. Chem Cent J, 2013. 7(1): p. 44. [PubMed: 23452343]
15. Yoo J and Aksimentiev A, New tricks for old dogs: improving the accuracy of biomolecular force fields by pair-specific corrections to non-bonded interactions. Phys Chem Chem Phys, 2018. 20(13): p. 8432–8449. [PubMed: 29547221]
16. Davis MR and Dougherty DA, Cation-pi interactions: computational analyses of the aromatic box motif and the fluorination strategy for experimental evaluation. Phys Chem Chem Phys, 2015. 17(43): p. 29262–70. [PubMed: 26467787]
17. Mecozzi S, West AP, and Dougherty DA, Cation-pi interactions in simple aromatics: Electrostatics provide a predictive tool. Journal of the American Chemical Society, 1996. 118(9): p. 2307–2308.
18. Bacher JM and Ellington AD, Selection and characterization of Escherichia coli variants capable of growth on an otherwise toxic tryptophan analogue. J Bacteriol, 2001. 183(18): p. 5414–25. [PubMed: 11514527]
19. Noren CJ, et al., A general method for site-specific incorporation of unnatural amino acids into proteins. Science, 1989. 244(4901): p. 182–8. [PubMed: 2649980]
20. Nowak MW, et al., Nicotinic receptor binding site probed with unnatural amino acid incorporation in intact cells. Science, 1995. 268(5209): p. 439–42. [PubMed: 7716551]

21. Saks ME, et al., An engineered Tetrahymena tRNAGln for in vivo incorporation of unnatural amino acids into proteins by nonsense suppression. The Journal of biological chemistry, 1996. 271(38): p. 23169–75. [PubMed: 8798511]
22. Infield DT, et al., Orthogonality of Pyrrolysine tRNA in the Xenopus oocyte. Sci Rep, 2018. 8(1): p. 5166. [PubMed: 29581437]
23. Ackerman MJ and Clapham DE, Ion channels--basic science and clinical disease [see comments] [published erratum appears in N Engl J Med 1997 Aug 21;337(8):579]. 1997. 336(22): p. 1575–1586.
24. Hattori M, Hibbs RE, and Gouaux E, A fluorescence-detection size-exclusion chromatography-based thermostability assay for membrane protein precrystallization screening. Structure, 2012. 20(8): p. 1293–9. [PubMed: 22884106]
25. Cheng Y, Membrane protein structural biology in the era of single particle cryo-EM. Curr Opin Struct Biol, 2018. 52: p. 58–63. [PubMed: 30219656]
26. Gallivan JP and Dougherty DA, Cation-pi interactions in structural biology. Proc Natl Acad Sci U S A, 1999. 96(17): p. 9459–64. [PubMed: 10449714]
27. Pless SA, et al., A cation-pi interaction in the binding site of the glycine receptor is mediated by a phenylalanine residue. J Neurosci, 2008. 28(43): p. 10937–42. [PubMed: 18945901]
28. Beene DL, et al., Cation-pi interactions in ligand recognition by serotonergic (5-HT3A) and nicotinic acetylcholine receptors: the anomalous binding properties of nicotine. Biochemistry, 2002. 41(32): p. 10262–9. [PubMed: 12162741]
29. Zhong W, et al., From ab initio quantum mechanics to molecular neurobiology: a cation-pi binding site in the nicotinic receptor. Proc Natl Acad Sci U S A, 1998. 95(21): p. 12088–93. [PubMed: 9770444]
30. Pless SA, et al., Molecular basis for class Ib anti-arrhythmic inhibition of cardiac sodium channels. Nat Commun, 2011. 2: p. 351. [PubMed: 21673672]
31. Ahern CA, et al., Electrostatic contributions of aromatic residues in the local anesthetic receptor of voltage-gated sodium channels. Circ Res, 2008. 102(1): p. 86–94. [PubMed: 17967784]
32. Lolicato M, et al., K2P2.1 (TREK-1)-activator complexes reveal a cryptic selectivity filter binding site. Nature, 2017. 547(7663): p. 364–368. [PubMed: 28693035]
33. Santarelli VP, et al., Calcium block of single sodium channels: role of a pore-lining aromatic residue. Biophys J, 2007. 93(7): p. 2341–9. [PubMed: 17545248]
34. Pan Y, et al., Cation-pi interaction regulates ligand-binding affinity and signaling of integrin alpha4beta7. Proc Natl Acad Sci U S A, 2010. 107(50): p. 21388–93. [PubMed: 21098296]
35. Santarelli VP, et al., A cation-pi interaction discriminates among sodium channels that are either sensitive or resistant to tetrodotoxin block. J Biol Chem, 2007. 282(11): p. 8044–51. [PubMed: 17237232]
36. Rahman MM, et al., Structure of the Native Muscle-type Nicotinic Receptor and Inhibition by Snake Venom Toxins. Neuron, 2020. 106(6): p. 952–962 e5. [PubMed: 32275860]
37. Yen TJ, et al., Structure of the saxiphilin:saxitoxin (STX) complex reveals a convergent molecular recognition strategy for paralytic toxins. Sci Adv, 2019. 5(6): p. eaax2650. [PubMed: 31223657]
38. Waheed Q, et al., Interfacial Aromatics Mediating Cation-pi Interactions with Choline-Containing Lipids Can Contribute as Much to Peripheral Protein Affinity for Membranes as Aromatics Inserted below the Phosphates. J Phys Chem Lett, 2019. 10(14): p. 3972–3977. [PubMed: 31246477]
39. Cheng J, et al., The cation-pi box is a specific phosphatidylcholine membrane targeting motif. J Biol Chem, 2013. 288(21): p. 14863–73. [PubMed: 23576432]
40. Dougherty DA and Stauffer DA, Acetylcholine binding by a synthetic receptor: implications for biological recognition. Science, 1990. 250(4987): p. 1558–60. [PubMed: 2274786]
41. Dougherty DA, Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. Science, 1996. 271(5246): p. 163–8. [PubMed: 8539615]
42. Scrutton NS and Raine AR, Cation-pi bonding and amino-aromatic interactions in the biomolecular recognition of substituted ammonium ligands. Biochem J, 1996. 319 ( Pt 1): p. 1–8. [PubMed: 8870640]

43. Sussman JL, et al., Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. Science, 1991. 253(5022): p. 872–9. [PubMed: 1678899]
44. Galzi JL and Changeux JP, Neuronal nicotinic receptors: molecular organization and regulations. Neuropharmacology, 1995. 34(6): p. 563–82. [PubMed: 7566492]
45. Brejc K, et al., Crystal structure of an Ach-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature, 2001. 411(6835): p. 269–76. [PubMed: 11357122]
46. Morales-Perez CL, Noviello CM, and Hibbs RE, X-ray structure of the human alpha4beta2 nicotinic receptor. Nature, 2016. 538(7625): p. 411–415. [PubMed: 27698419]
47. Padgett CL, et al., 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, 2007. 27(4): p. 886–92. [PubMed: 17251430]
48. Lummis SC, et al., Two amino acid residues contribute to a cation-pi binding interaction in the binding site of an insect GABA receptor. J Neurosci, 2011. 31(34): p. 12371–6. [PubMed: 21865479]
49. Ahern CA, et al., The hitchhiker's guide to the voltage-gated sodium channel galaxy. J Gen Physiol, 2016. 147(1): p. 1–24. [PubMed: 26712848]
50. Savio-Galimberti E, Argenziano M, and Antzelevitch C, Cardiac Arrhythmias Related to Sodium Channel Dysfunction. Handb Exp Pharmacol, 2018. 246: p. 331–354. [PubMed: 28965168]
51. Kaplan DI, Isom LL, and Petrou S, Role of Sodium Channels in Epilepsy. Cold Spring Harb Perspect Med, 2016. 6(6).
52. Dib-Hajj SD, et al., Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. Brain, 2005. 128(Pt 8): p. 1847–54. [PubMed: 15958509]
53. Ragsdale DS, et al., Molecular determinants of state-dependent block of Na+ channels by local anesthetics. Science, 1994. 265(5179): p. 1724–8. [PubMed: 8085162]
54. Carboni M, et al., Slow sodium channel inactivation and use-dependent block modulated by the same domain IV S6 residue. J Membr Biol, 2005. 207(2): p. 107–17. [PubMed: 16477531]
55. Ragsdale DS, et al., Common molecular determinants of local anesthetic, antiarrhythmic, and anticonvulsant block of voltage-gated Na+ channels. Proc Natl Acad Sci U S A, 1996. 93(17): p. 9270–5. [PubMed: 8799190]
56. Geffeney SL, et al., Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature, 2005. 434(7034): p. 759–63. [PubMed: 15815629]
57. Duran-Riveroll LM and Cembella AD, Guanidinium Toxins and Their Interactions with Voltage-Gated Sodium Ion Channels. Mar Drugs, 2017. 15(10).
58. Sivilotti L, et al., A single serine residue confers tetrodotoxin insensitivity on the rat sensory-neuron-specific sodium channel SNS. FEBS Lett, 1997. 409(1): p. 49–52. [PubMed: 9199502]
59. Shen H, et al., Structures of human Nav1.7 channel in complex with auxiliary subunits and animal toxins. Science, 2019. 363(6433): p. 1303–1308. [PubMed: 30765606]
60. Penzotti JL, et al., Differences in saxitoxin and tetrodotoxin binding revealed by mutagenesis of the Na+ channel outer vestibule. Biophys J, 1998. 75(6): p. 2647–57. [PubMed: 9826589]
61. Mahar J, et al., Pharmacological and biochemical properties of saxiphilin, a soluble saxitoxin-binding protein from the bullfrog (Rana catesbeiana). Toxicon, 1991. 29(1): p. 53–71. [PubMed: 1851343]
62. Hauser AS, et al., Trends in GPCR drug discovery: new agents, targets and indications. Nat Rev Drug Discov, 2017. 16(12): p. 829–842. [PubMed: 29075003]
63. Yang D, et al., G protein-coupled receptors: structure- and function-based drug discovery. Signal Transduct Target Ther, 2021. 6(1): p. 7. [PubMed: 33414387]
64. Kruse AC, et al., Activation and allosteric modulation of a muscarinic acetylcholine receptor. Nature, 2013. 504(7478): p. 101–6. [PubMed: 24256733]
65. Dror RO, et al., Structural basis for modulation of a G-protein-coupled receptor by allosteric drugs. Nature, 2013. 503(7475): p. 295–9. [PubMed: 24121438]
66. Kunishima N, et al., Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature, 2000. 407(6807): p. 971–7. [PubMed: 11069170]

67. Tsuchiya D, et al., Structural views of the ligand-binding cores of a metabotropic glutamate receptor complexed with an antagonist and both glutamate and Gd3+. Proc Natl Acad Sci U S A, 2002. 99(5): p. 2660–5. [PubMed: 11867751]

68. Bessis AS, et al., Closure of the Venus flytrap module of mGlu8 receptor and the activation process: Insights from mutations converting antagonists into agonists. Proc Natl Acad Sci U S A, 2002. 99(17): p. 11097–102. [PubMed: 12151600]

69. de March CA, et al., G protein-coupled odorant receptors: From sequence to structure. Protein Sci, 2015. 24(9): p. 1543–8. [PubMed: 26044705]

70. Zhu J, et al., Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. Mol Cell, 2008. 32(6): p. 849–61. [PubMed: 19111664]

71. Kang W, McNaughton RL, and Espinosa HD, Micro- and Nanoscale Technologies for Delivery into Adherent Cells. Trends Biotechnol, 2016. 34(8): p. 665–678. [PubMed: 27287927]

72. Lee YJ, et al., Genetically encoded fluorophenylalanines enable insights into the recognition of lysine trimethylation by an epigenetic reader. Chem Commun (Camb), 2016. 52(85): p. 12606–12609. [PubMed: 27711380]

## HIGHLIGHTS

Infield et al. “Cation-π interactions and their functional roles in membrane proteins”Ion Channel Special Edition

- The physicochemical characteristics of cation-π interactions are summarized.
- Cation- π abundance is assessed via automated analysis of 203 ion channels and 99 unique GPCR protein structures.
- Aromatic fluorination as a method to assess cation-π interactions is discussed in tandem with unnatural amino acid mutagenesis techniques.
- Examples of cation- π interactions are highlighted for sodium channels, acetylcholine receptors, saxiphilin, GPCRs, and, integrins.

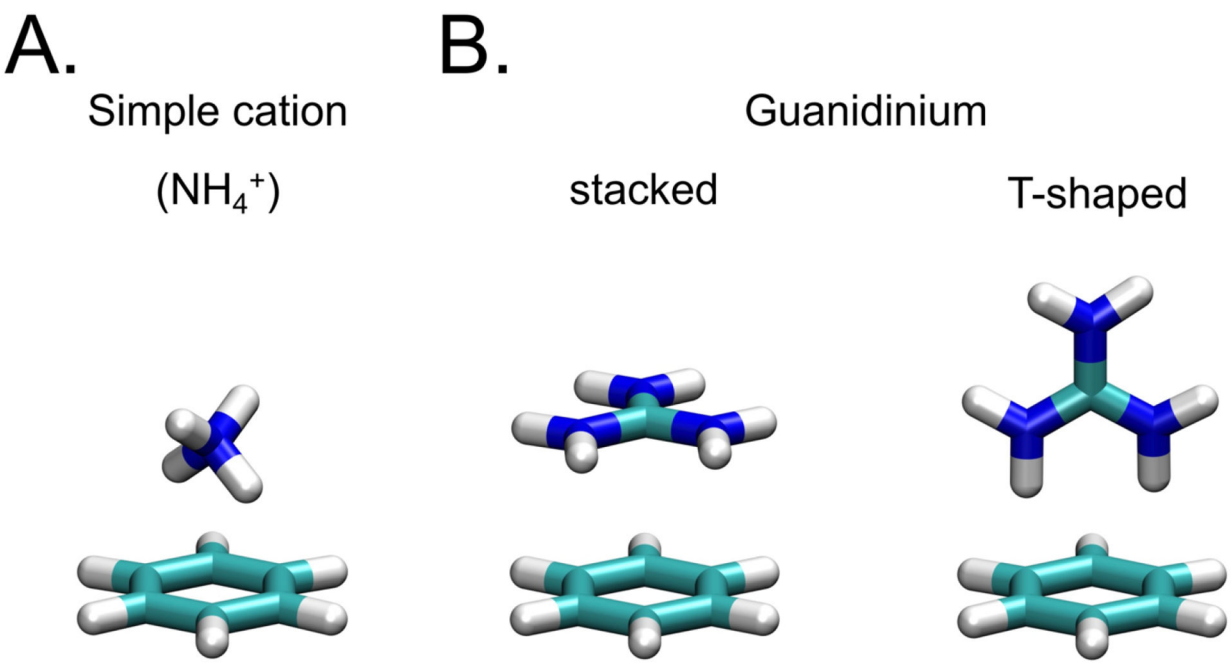



**Figure 1:**

Idealized geometries for cation-π interactions. **A)** Ideal position and distance for the interaction of sodium and benzene. **B**) Depiction of ideal position and distance for stacked (left) and t-shaped (right) interaction of guanidinium with benzene.

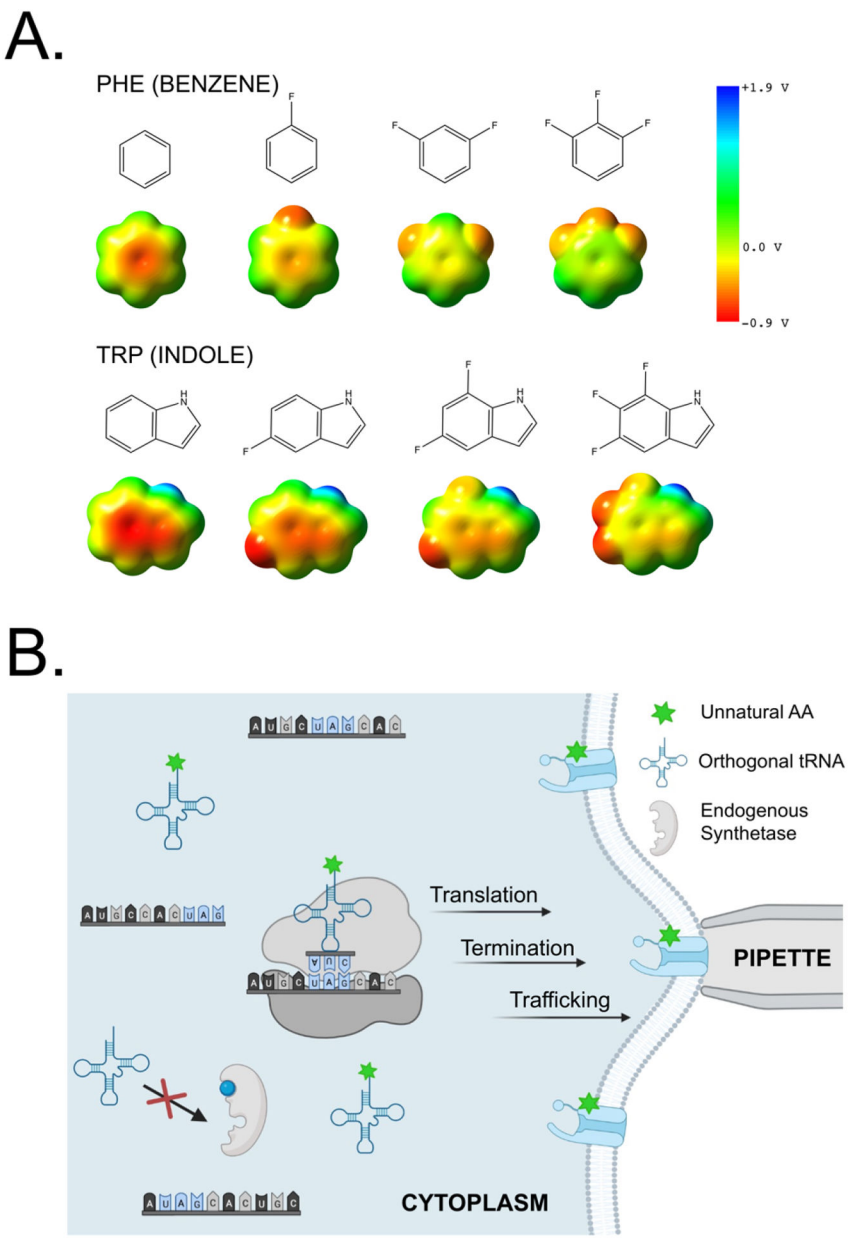



**Figure 2:**

**A)** effect of serial fluorination on the electrostatic potential of benzene and indole, structural surrogates for phenylalanine and tryptophan, respectively. **B)** Schematic view of *in vivo* encoding of unnatural amino acids via injection of chemically acylated orthogonal suppressor tRNA. Unacylated suppressor tRNA is not recognized by endogenous aminoacyl-tRNA synthetases (bottom left), while the acylated tRNA is competent for translation, specifically enabling the encoding of an unnatural amino acid in response to the amber stop codon (center). The engineered protein (in this case an ion channel) is trafficked to the plasma membrane where it can be assayed via electrophysiology.

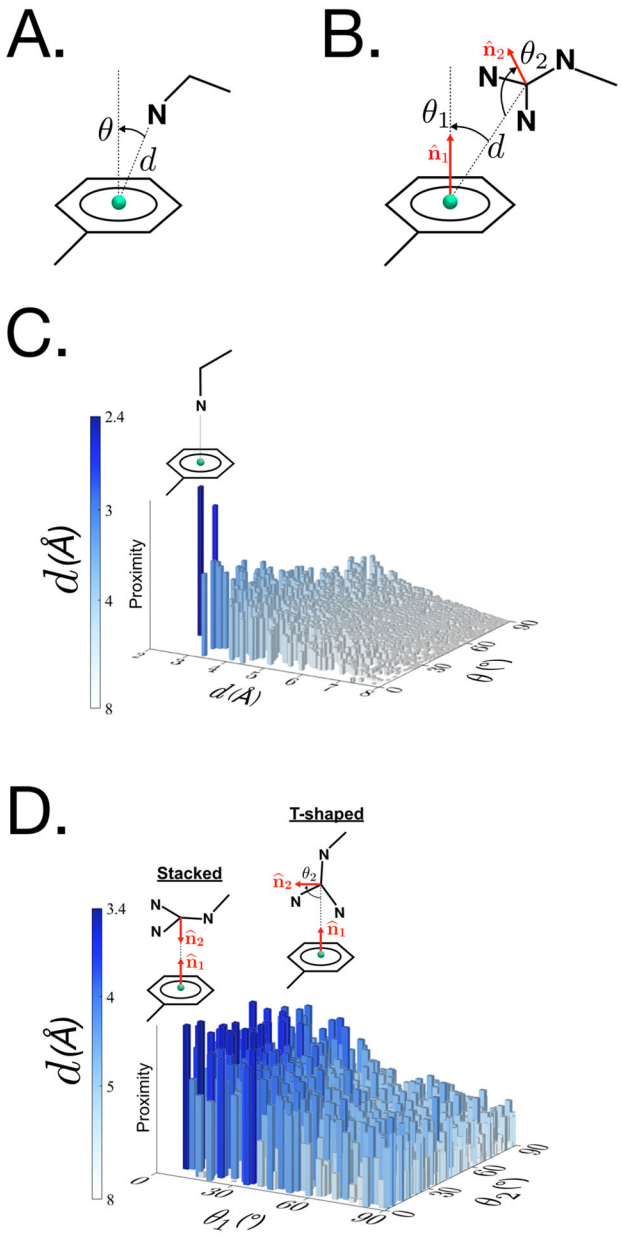



**Figure 3:**

Cation-π interactions naturally occurring in membrane channels and G protein coupled receptors. Geometric variables utilized to characterize the approach of **A)** the primary amine of lysine and **B)** the guanidinium moiety of arginine towards the plane of the aromatic side chain. *d* is the separation of the aromatic-ring centroid from either the nitrogen of the lysine side chain, or the ζ-carbon of the arginine side chain. θ is the angle formed by the normal to the plane of the aromatic ring and the unitary vector pointing from its centroid to the nitrogen of lysine. $\theta_1$ and $\theta_2$ are, respectively, the angle between the normal to the aromatic ring ($\hat{n}_1$) and the unitary vector pointing from its centroid toward the ζ-carbon of arginine, and the angle formed by the normal of the guanidinium moiety ($\hat{n}_2$) and the unitary vector pointing from its ζ-carbon atom toward the aromatic-ring centroid. **C)** Angular distribution

characterizing the approach of the primary amine of lysine toward phenylalanine, tyrosine, and tryptophan side chains of membrane channels and GPCRs. Taller columns denote closer proximity of the primary amine group from the plane of the aromatic ring. A structural depiction of the ideal angle of approach hovers over the plot near the closest interactions of lowest angular deviation. **D)** Correlation plot of the angles $\theta_1$ and $\theta_2$ of the guanidinium moiety of arginine from aromatic side chains in membrane channels and GPCRs, depicted as in C). Idealized structural depictions hover over stacked and t-shaped variations.

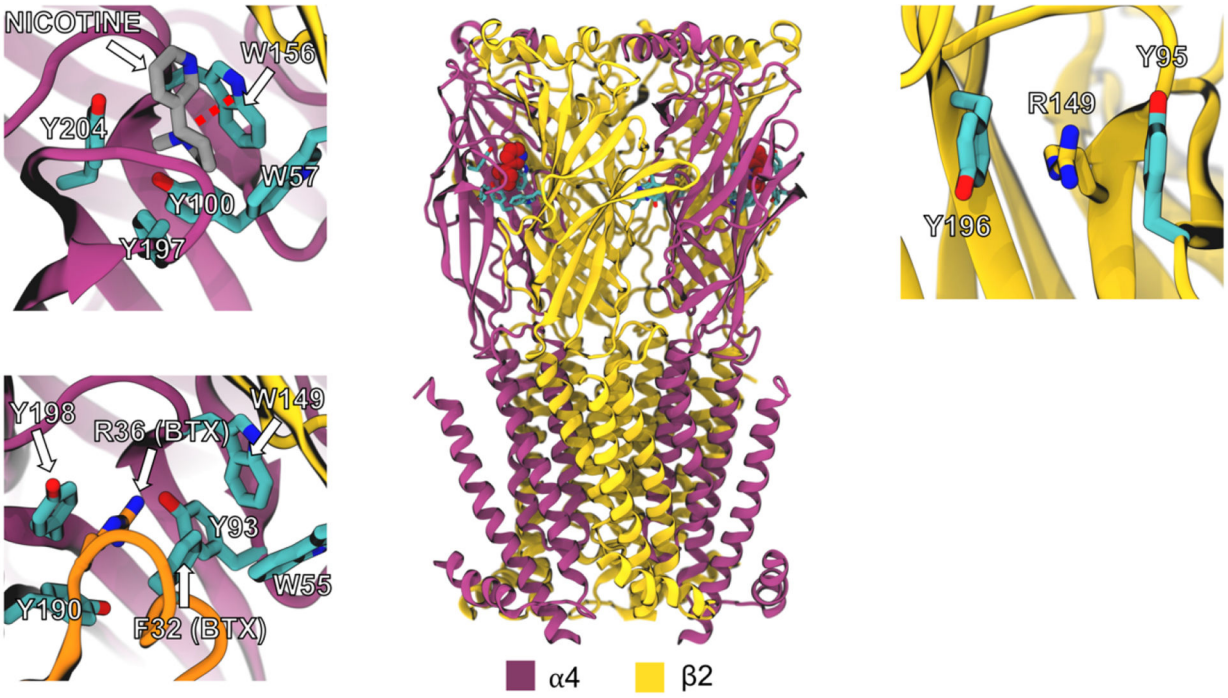



**Figure 4:**

Cation-π interactions in the nicotinic acetylcholine receptor (nAChR). Overall structure of the human α4/β2 nAChR is in the center, with nicotine ligand shown in red spheres. At top left is a closeup view of nicotine in its binding site. Of the aromatic residues comprising an aromatic box (cyan) around the ligand (gray), W156 (labeled) of α4 has been shown to engage in a cation-π interaction with the ligand (red dashed line). At top right is a closeup view of a structural cation-π within β2 found in non-ligand binding interfaces. Relevant aromatic residues are shown in cyan. At bottom left is a closeup view from the cryo-EM structure of the native *Torpedo* nAChR bound to bungarotoxin (orange), showing a cation-π interaction between Y198 (labeled) on the nAChR α subunit (analogous to Y204 in the human α4) and an arginine/phenylalanine pair from bungarotoxin (labeled).

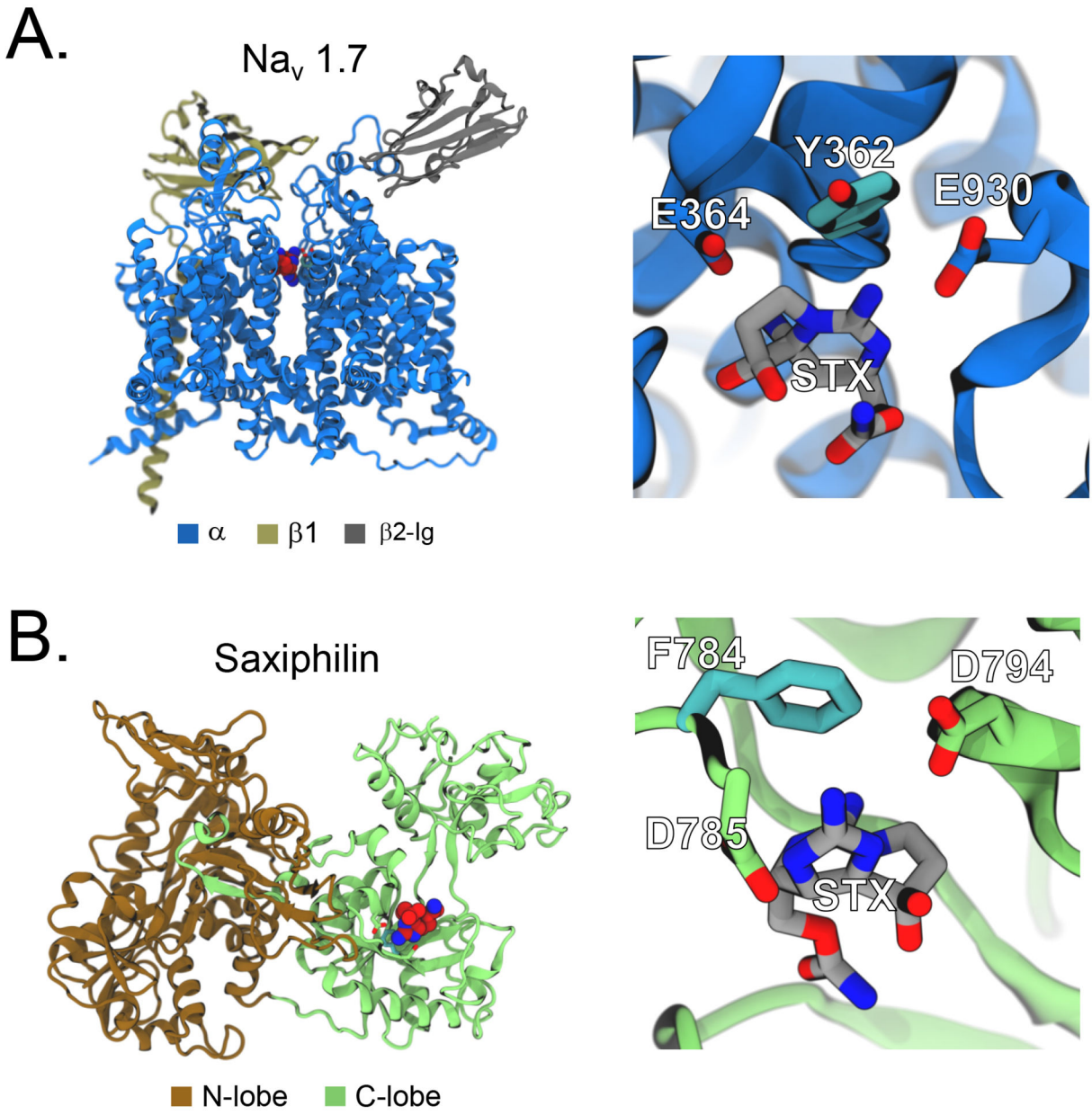



**Figure 5:**

Saxitoxin (STX) binding mediated by cation-π interactions with conserved aromatic residues in two different proteins. **A)** Left, overall Cryo-EM structure of hNa$_v$ 1.7 bound to STX (shown in red spheres), shown with the resolvable regions of the β1 and β2 auxiliary subunits. At right is a close up (top-down view) showing a cation-π interaction with the toxin as well as nearby interactions with acidic residues. **B)** Left, crystal structure of bullfrog saxiphillin bound to STX (shown in red spheres), with close up at right showing a chemically similar structural motif to that seen in the Na$_v$

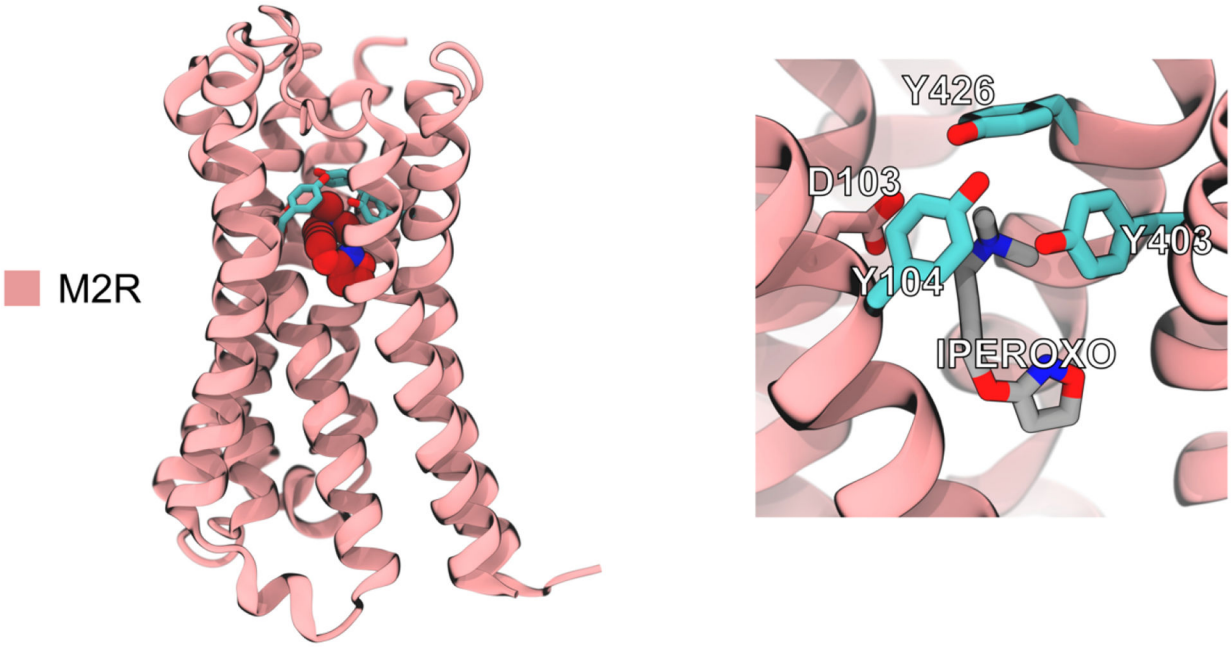



**Figure 6:**

Cation-π interactions in the orthosteric binding site of the M2 acetylcholine receptor (M2R). Left, overall structure of M2R (salmon) bound to the orthosteric ligand iperoxo (red spheres). Right, closeup view of the iperoxo binding site, with tyrosine residues involved in a cation-π interaction shown in cyan.