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Interacting Amino Acid Replacements Allow Poison Frogs to Evolve Epibatidine Resistance

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Abstract

Animals that wield toxins face self-intoxication. Poison frogs have a diverse arsenal of defensive alkaloids that target the nervous system. Among them is epibatidine, a nicotinic acetylcholine receptor (nAChR) agonist that is lethal at microgram doses. Epibatidine shares a highly conserved binding site with acetylcholine, making it difficult to evolve resistance yet maintain nAChR function. Electrophysiological assays of human and frog nAChR revealed that one amino acid replacement, which evolved three times in poison frogs, decreased epibatidine sensitivity but at a cost of acetylcholine sensitivity. However, receptor functionality was rescued by additional amino acid replacements that differed among poison frog lineages. Our results demonstrate how resistance to agonist toxins can evolve and that such genetic changes propel organisms towards an adaptive peak of chemical defense.

Acquiring chemicals from the environment and recycling them for anti-predator defense is a survival strategy that has evolved in nearly every major branch of life (1). Exposure to toxic chemicals may have high physiological costs, but it can also be an opportunity for organisms to capitalize on these substances as new resources. Organisms that accumulate these chemicals risk self-intoxication unless they can resist their own defenses through compartmentalization, metabolic detoxification, or target-site insensitivity, i.e., changes in the molecular target of the toxin that affect its ability to bind (2). Many toxins target evolutionarily conserved proteins such as ion channels, which govern key nervous system functions. Thus, revealing the mechanistic basis of toxin resistance deepens our understanding of protein function and provides insights into nervous system evolution (3, 4).

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Moreover, the physiology of toxin resistance is a crucial aspect of chemical defense and characterizing the evolution of resistance might elucidate how and why organisms acquire toxic defenses (5).

Neotropical poison frogs (Dendrobatidae) have independently evolved chemical defenses at least four times (6). The origins of chemical defense are usually accompanied by shifts towards bright coloration, resulting in a complex phenotype or syndrome known as aposematism (6). Theoretically, aposematic and non-aposematic poison frogs represent alternative peaks on an adaptive landscape that arose as a result of disruptive selection that favored more extreme phenotypes over intermediate ones (e.g., conspicuous but not well defended, or defended but not aposematic) (7). The multiple origins of aposematism within dendrobatids suggest that the switch from non-aposematic to aposematic phenotypes is easily attained within this group. Characterizing the evolution of toxin resistance, a key step in this phenotypic transition, may reveal pathways between these adaptive peaks in which toxin resistance facilitates origins of toxin sequestration.

Chemically defended dendrobatids take up from their diet over 800 types of lipophilic alkaloids (8), many of which modulate nervous system function (9). Their effects vary from benign to lethal (10), but most are bitter-tasting and thus generally aversive to predators (11). Epibatidine, one of the best known of these alkaloids, was first isolated from the phantasmal poison frog *Epipedobates anthonyi* in 1974 (12). Epibatidine has an analgesic effect 200 times that of morphine, yet it targets a specific subset of nicotinic acetylcholine receptors (nAChRs) rather than opioid receptors (13). Because of these qualities, epibatidine has inspired pharmacological innovations, although its toxicity has prohibited its successful development as a pharmaceutical (14).

Toxic animals, including poison frogs, often evolve resistance to their toxins via amino acid (AA) replacements in toxin-binding sites (target-site insensitivity; 15, 16). The location of these replacements is constrained by protein function, leading to predictable and convergent mechanisms of resistance (17). For example, resistance to tetrodotoxin (TTX), a Na_V1 voltage-gated sodium channel blocker, evolved many times in toxic pufferfish, newts, and snakes that feed on newts via various AA replacements at residues in Na_V1 proteins that interact with TTX (17–19). Similarly, resistance to cardiac glycosides, which inhibit the sodium-potassium pump, has evolved at least fourteen times in toxic insects and amphibians as well as their predators via AA replacements in the cardiac-glycoside binding site (4, 20).

Evolving epibatidine resistance involves different strategies at the molecular level, as epibatidine is an agonist that shares a binding site with ACh, the endogenous ligand of nAChRs, while TTX and cardiac glycosides act on receptors that are not ligand-gated (21, 22). Resistance to epibatidine thus requires decreased sensitivity to epibatidine while preserving sensitivity to the endogenous agonist ACh that interacts with many of the same AAs, all without disrupting the normal receptor function.

Phylogenetic identification of AA replacements in the poison frog nAChR

Based on what is known about the toxin and ligand, we hypothesized that epibatidine-bearing frogs would have nAChRs that resist epibatidine yet display normal ACh sensitivity, and that the basis of resistance would involve genetic changes in the ligand-binding site. To test this hypothesis, we sequenced genes in poison frogs encoding the primary molecular target of epibatidine in the brain, the α4β2 nAChR (chrna4 and chrnb2) (23). Epibatidine has been detected in two distinct lineages of dendrobatids, *Epipedobates* and *Ameerega* (12), so we predicted two origins of resistance. Consequently, we sequenced 9 species of these genera as well as 19 other species of poison frogs, including 8 species of Dendrobatinae (*Dendrobates+Phyllobates*), a clade of chemically defended poison frogs lacking epibatidine, and 11 non-defended species (table S1, 24).

Four sites in the β2 subunit (F106, S108, A110, and I118, numeration of the mature human protein) have unique AA replacements in the alkaloid-sequestering dendrobatids *Epipedobates, Ameerega*, and *Dendrobates* (subgenus *Oophaga sensu 25*; Fig. 1A), the last of which is not known to have epibatidine defenses. These replacements are near the epibatidine-binding site in the α^{+}–β^{-} interface: between loops A and E, and in loop E (Fig. 1B–E) (21, 22). Each of these replacements involves a single nucleotide change in the first or second codon position (table S2, 24), suggesting non-neutral evolution. Five additional sites in α4 were found to have AA replacements unique to these poison frogs, but only one of these (D176N) was near the epibatidine-binding site (table S3 and fig. S1, 24).

Electrophysiology of AA replacements in the poison frog nAChR

The α4β2 nAChR is a pentameric protein that exhibits two different stoichiometries: a high-ACh sensitivity conformation (HS), $(\alpha 4)_2(\beta 2)_3$, and a low-ACh sensitivity conformation (LS), $(\alpha 4)_3(\beta 2)_2$ (26, 27). To determine experimentally whether the identified AA replacements provide resistance to epibatidine, we used site-directed mutagenesis to introduce poison frog AA replacements into human nAChRs. We then co-expressed the wild-type and mutated β2 subunits with human α4 nAChR subunits in *Xenopus laevis* oocytes and measured acetylcholine and epibatidine concentration-response curves (CRCs) through two-electrode voltage clamp (parameters, results, and statistical analyses from all CRCs are shown in tables S5–S16, 24). For each subunit combination, we injected different ratios of α4 and β2 transcripts to favor the formation of either HS or LS conformations (24). For brevity, we describe only HS nAChRs in the main text, as we found the same general pattern of channel sensitivity to ACh and epibatidine in both stoichiometries. For LS nAChR results see fig. S2 and tables S5–S7 (24). We also performed electrophysiology experiments to test whether the one replacement in the α4 subunit near the ligand-binding site (D176N) affected LS nAChR function, but we found no evidence for an effect (24; fig. S1 and S4, table S8).

For clarity, we denote all nAChR genotypes with four letters indicating the AA residue at each of the four sites of interest (106, 108, 110, and 118, see Table 1). Bold letters in each genotype indicate AA replacements introduced into a transcript via site-directed mutagenesis.

Human-to-frog mutants

The *Epipedobates* and *Ameerega* replacement patterns (**LC**AI and F**CVV** genotypes) produced by mutagenesis showed ACh concentration-response curves (CRCs) identical to that of wild-type human FSAI genotype, while the subgenus *Oophaga* replacement pattern (F**C**AI) showed a decrease in sensitivity to ACh (Fig. 2A). All three nAChRs with poison frog AA replacement patterns (**LC**AI, F**CVV**, F**C**AI) were less sensitive to epibatidine than the wild-type receptor (Fig. 2B), indicating that these replacement patterns are sufficient to produce epibatidine-resistant phenotypes (Tables 1, S5−S7; 24). Interestingly, the ACh CRC is biphasic for the *Oophaga* replacement pattern, suggesting that in the human genetic background the S108C replacement may induce assembly of low-sensitivity (LS) nAChRs. As the LS stoichiometry possess two kinds of binding sites, application of increasing concentration of ACh results in a biphasic curve that reflects activation of the two HS binding sites at low ACh concentrations and of the single LS binding site at high ACh concentrations (24). Thus, resistance to epibatidine conferred by the S108C replacement incurs a cost of ACh sensitivity in the human β2 subunit.

We then characterized the physiological effect of each individual replacement in poison frogs by generating human α4β2 nAChR transcripts with single amino acid replacements (**L**SAI, FS**V**I, and FSA**V**). As with the S108C replacement, human transcripts with the I118V replacement (FSA**V**, derived in *Ameerega*) provided moderate resistance to epibatidine at a cost of ACh sensitivity, possibly because this AA replacement also induced assembly of LS nAChRs (Fig. 2C and D, Table 1). In contrast, human receptors with either F106L (*Epipedobates*) or A110V (*Ameerega*) displayed no change in ACh and epibatidine sensitivity, indicating that these replacements probably do not contribute to epibatidine resistance (Fig. 2E to H, Table 1). Instead, these replacements appear to compensate for the decrease in ACh sensitivity incurred by the replacements that provided resistance (Table 1), as human receptors with the **LC**AI genotype (*Epipedobates*) or the F**CVV** genotype (*Ameerega*) both showed normal ACh response (Fig. 2A and B; Table 1; 24).

Epipedobates-to-human mutants

We synthesized and expressed the wild-type *Epipedobates* α4β2 nAChR (LCAI genotype) and a double mutant replicating the plesiomorphic human genotype (**FS**AI) in *Xenopus laevis* oocytes, and performed electrophysiology assays. The *Epipedobates*-to-human mutant (**FS**AI**)** showed greatly increased sensitivity to epibatidine but no change in sensitivity to ACh (Table 1 and S7; Fig. 2G and H) indicating that the replacements in *Epipedobates* were necessary for resistance.

To understand the contributions of each replacement when it occurs in the poison frog genetic background, we expressed the single mutant genotypes **F**CAI and L**S**AI in the *Epipedobates* β2 subunit. While S108C incurred a drastic cost in ACh sensitivity in the human genetic background (Fig. 2A, compare FSAI and F**C**AI), the *Epipedobates*-to-human **F**CAI mutant demonstrated only a minor (but significant) decrease in sensitivity to ACh (compared to **FS**AI), suggesting that some other aspects of the poison frog genetic background ameliorate the large cost of this replacement in the human F**C**AI genotype (Table 1). This difference may be explained by the observation that the S108C replacement in human receptors appeared to induce formation of LS nAChRs (Fig. 2A), which are less sensitive to ACh than HS nAChRs. However, the *Epipedobates* nAChR never appeared to form the LS stoichiometry, even when the injected cRNA subunit ratio favored its formation (compare Fig. 2G and H to fig. S2G and H, 24). Little is known about the poison frog α4β2 nAChR, but the apparent absence of the LS stoichiometry in *Epipedobates* (evidenced by the lack of a biphasic, right-shifted curve) lessens the cost of the S108C replacement, and might be related to epibatidine exposure and resistance.

As predicted, the *Epipedobates* **F**CAI receptor displayed a decrease in sensitivity to epibatidine compared with **FS**AI (Tables 1 and S7; 24), confirming the role of S108C in epibatidine resistance (Fig. 2G and H). As with the human-to-frog **L**SAI receptor, the *Epipedobates*-to-human L**S**AI receptor affected neither ACh nor epibatidine sensitivities compared to **FS**AI (Table 1). The LCAI genotype (wild-type in *Epipedobates*) displayed normal responses to ACh and decreased sensitivity to epibatidine (Table 1). Thus, as in the human receptor, C108 provides epibatidine resistance and L106 appears to compensate by normalizing α4β2 receptor function in *Epipedobates* poison frogs.

AA replacements in poison frog nAChR are proximal to the epibatidine binding site

We found that AA replacements in the poison frog β2 subunit (Fig. 1) alter α4β2 nAChR sensitivity to epibatidine (Fig. 2B). We propose that this is in part due to the proximity of the AA replacements to the epibatidine binding site. Namely, the β2C108 residue directly contacts the side chain of α4W156, one of the main determinants in stabilizing epibatidine binding (Fig. 1D and E) (21, 28). The sulfur-containing side chain of C108 is bulkier than that of serine, and it could modify the epibatidine-W156 interaction. The I118V replacement in *Ameerega*, which also contributes to epibatidine resistance (Fig. 1D and E), is next to F119, a residue that interacts with the epibatidine chloropyridine ring and stabilizes the epibatidine chlorine atom through its backbone carboxyl group. Moreover, the A110V replacement is next to V111, another AA residue that interacts with epibatidine via van der Waals forces (21, 28, 29). These replacements are located in β-sheets that are involved in epibatidine binding, but are less involved in ACh binding (21, 28, 30). The $\beta 2^{-}$ side of the binding pocket is further from ACh than is the $\alpha 4^{+}$ side, and thus forms looser interactions with ACh, such that AA replacements in the β^{-} region that allow changes in epibatidine binding may be less likely to affect ACh sensitivity. This structure-function problem was apparently solved via an identical genetic change three times within poison frogs and refined via different genetic changes at least twice in these lineages.

Evolutionary pathways towards epibatidine resistance

Toxin resistance often evolves in response to recurrent exposure to toxins (2, 4, 31, 32); thus patterns of resistance should reflect the evolutionary history of toxin exposure. The evolutionary patterns of AA replacements in the poison frog β2 nAChR subunit suggest that in each of the *Epipedobates, Ameerega*, and *Dendrobates* (*Oophaga*) clades (Fig. 1A), an ancestral species was likely exposed to epibatidine, resulting in selection for and evolution of epibatidine resistance approximately 5, 10, and 8MYA, respectively (25). Although no clade of poison frogs that has epibatidine defense lacks AA replacements in the β2 nAChR, epibatidine has only been detected in two of three sampled species of *Epipedobates*, in two of twelve sampled species of *Ameerega*, and in none of nine sampled species of *Dendrobates* (*Oophaga*) (9, 12). It is possible that some populations with epibatidine defense are extinct or have not been detected, or that the dietary source of epibatidine, presumed to be an arthropod, is not as available as it was long ago (12). While epibatidine resistance may have arisen as a side effect of some other change to the protein, mutations in the ligand-binding domain are uncommon (Fig. 1A) and presumably evolve under strong selective pressures. Regardless of the apparent rarity of epibatidine in poison frogs, the epibatidine-resistant phenotype (determined by electrophysiology) does not appear to have been lost in any resistant lineages (*Oophaga*, *Epipedobates*, or *Ameerega*), suggesting that lack of resistance has a high cost, that reversion to a non-resistant phenotype is physiologically difficult, or that maintenance of epibatidine resistance is not costly.

The evolutionary patterns underlying origins of epibatidine resistance in poison frogs reflect an adaptive landscape with two peaks that maximize fitness of alternative phenotypes: toxin-resistant and defended or toxin-sensitive and undefended. Given that S108C provides significant epibatidine resistance and that it is found in all three resistant clades, we argue that it provides a substantial selective advantage. We suggest two possible evolutionary pathways for acquisition of toxin resistance. In the first, initial replacements may provide a small selective benefit of resistance yet carry some physiological cost in receptor function. For example, the S108C replacement arose independently in all three lineages and is sufficient to produce an epibatidine-resistance phenotype. However, it also incurs decreased sensitivity to ACh in both the human and the *Epipedobates* backgrounds (Table 1), and the fitness cost of this replacement in living organisms is not clear. We speculate that yet unidentified mutants in the poison frog nAChR sustained receptor functionality, i.e., by inducing nAChR expression changes, until other replacements such as F106L evolved to rescue receptor sensitivity to ACh. Disruptive selection on populations with both genotypes may have propelled the populations with S108C toward a new adaptive peak.

In the second possible trajectory, certain mutants already present in the gene pool provide a genetic background in which resistance arises without cost (e.g., F106). For example, the artificial genotype **L**SAI (F106L) shows no reduction in either ACh or epibatidine sensitivity (Table 1). Thus, a frog species with F106L has evolved a novel genotype (LSAI), intermediate between FSAI (plesiomorphic) and LCAI in *Epipedobates*, without incurring a cost, which subsequently allows the C108 replacement to also evolve without cost. However, the LSAI genotype does not exist in any taxa we sampled. It is not present in *Silverstoneia*, the sister-group of *Epipedobates* (two of eight species sampled), nor in the closely related taxa *Ameerega* and *Colostethus* (Fig. 1A). Thus, this second pathway, in which a novel genotype evolves without apparent cost, is not found in poison frogs. However, this pathway is known in the brown plant-hopper (*Nilaparvata lugens*) (33), in which two AA replacements confer resistance to fipronil, a noncompetitive antagonist of GABA receptors. This occurs in an apparently sequential process where the second AA change provides high resistance yet has a high fitness cost and never occurs without the first (33). It is unclear how common such pre-existing compensatory mutations are, although it appears that mutations providing incremental increases in resistance are quite common. In Danainae butterflies, newts, garter snakes, and poison frogs, toxin resistance tends to increase over evolutionary time via additional AA replacements that occur in parallel with increased concentrations of chemical defenses (15, 16, 34, 35). It is possible that pre-adaptive mutations that allow resistance to evolve with little cost are present in these organisms and simply have not been identified. The presence of such pre-adaptive mechanisms would imply a shallow, "neutral" valley on the adaptive landscape that facilitates the movement from one adaptive peak to another.

The *Epipedobates, Ameerega*, and *Dendrobates* (*Oophaga*) clades, which are evolutionarily young (6, 36), are an example of rapid and ongoing diversification possibly driven by the evolution of resistance to anti-predator toxins (15). We demonstrate that resistance to epibatidine involves finely tuning a highly conserved binding site without disrupting receptor function, providing insights into evolutionary pathways culminating in chemical defenses. Thus, evolution, with millions of years and subjects, can solve complex problems in systems biology that may otherwise seem impossible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One Sentence Summary

Mutations combine to confer epibatidine resistance without altering acetylcholine response in a poison frog nicotinic acetylcholine receptor.

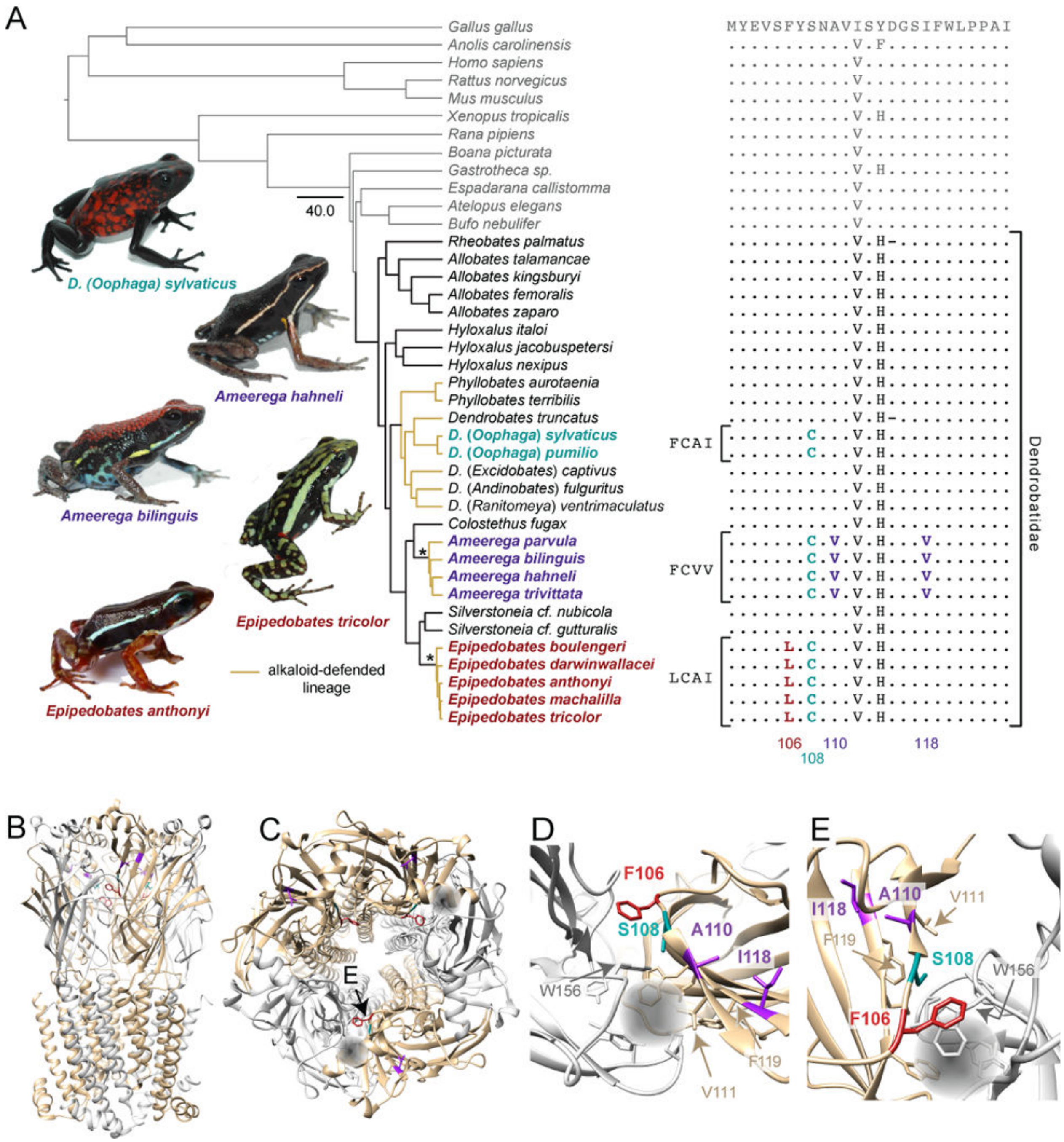

Fig. 1. AA replacements in β2 associated with alkaloid-defended poison frogs

(**A**)

Alignment of dendrobatid (black), non-dendrobatid (grey), and outgroup (grey) β2 sequences (table S4). Yellow branches in the phylogeny (adapted from 36) indicate alkaloid-defended lineages; asterisks indicate clades in which epibatidine has been detected; the unit of the scale bar is number of expected substitutions per site. Focal species names are in bold and colored by their AA replacement pattern. The AA replaced only in *Epipedobates* poison frogs is in red (F106L); AAs replaced only in *Ameerega* are in purple (A110I and I118V);

the convergently evolved replacement is in cyan (S108). Genotypes of clades with replacements are indicated to the left of the alignment (see Table 1). Structure of the human $(\alpha 4)_2(\beta 2)_3$ nAChR (22) (**B**) from the side and (**C**) from extracellular space. α4 subunits are in light grey, β2 subunits are in gold, and the ligand-binding sites are indicated by grey spheres. AA residues identified with grey and gold arrows are known to be involved in ACh and/or epibatidine binding (21, 28, 29). Closer view of the binding site from (**D**) extracellular space and (**E**) viewpoint indicated by labeled arrow in (**C**).

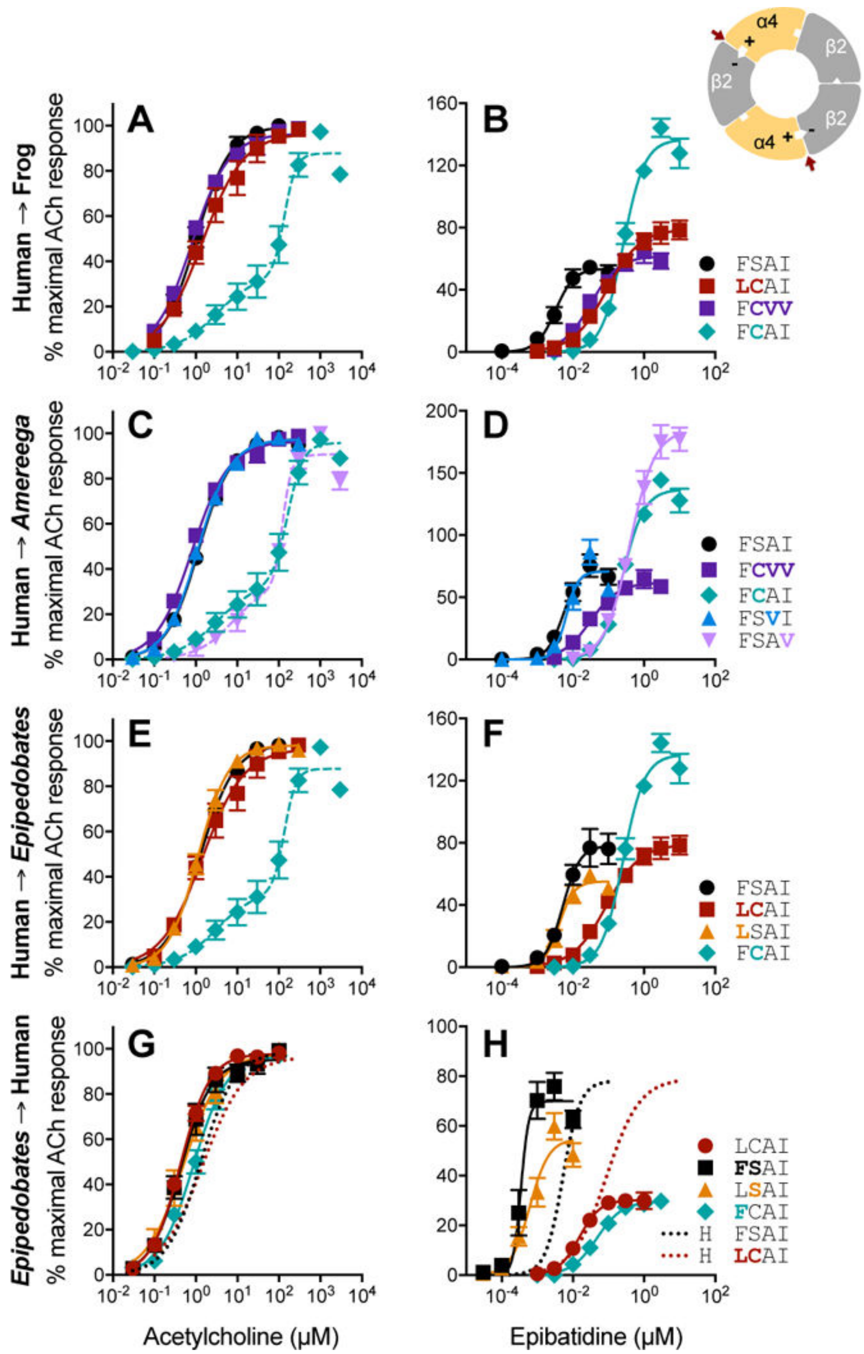

Fig. 2. ACh and epibatidine concentration-response curves in high-sensitivity α4β2 nAChRs

Left panels show responses to ACh and right panels show responses to epibatidine. (**A**, **B**) Human α4β2 nAChRs: wild-type genotype (FSAI) and receptors containing the AA patterns identified in *Epipedobates*, *Ameerega*, and *Dendrobates* (*Oophaga*) poison frogs (**LC**AI, F**CVV**, and F**C**AI genotypes, respectively). (**C**, **D**) Human α4β2 nAChRs: wild-type (FSAI) and *Ameerega* genotypes (F**CVV**, F**C**AI, FS**V**I and FSA**V**). (**E**, **F**) Human α4β2 nAChRs: wild-type (FSAI) and *Epipedobates* genotypes (**LC**AI, **L**SAI and F**C**AI). (**G**, **H**) *Epipedobates* α4β2 nAChRs: wild-type (LCAI genotype) and human genotypes (**FS**AI,

FCAI and L**S**AI). Dotted lines (⋯) correspond to human FSAI and LCAI curves from C and D panels. Error bars smaller than the symbols are not visible. Data were fitted to either mono- (−) or biphasic (–) curves. Inset: schematic of HS α4β2 nAChR stoichiometry; ligand-binding sites indicated by arrows.

Table 1

Effects of AA replacements on ligand responses in human and *Epipedobates* high-ACh-sensitivity (HS) nAChRs

Relative fold change in sensitivity induced by AA replacements was calculated as [mutant EC_{50}]/[FSAI EC_{50}] for each genetic background (i.e., [mutant]/[reference]; see tables S5−S7; 24). Values greater than 1 indicate that relatively more ligand is required to elicit the same response; thus, higher values indicate lower sensitivity. ACh assessments for biphasic curves (>1) are qualitative, but both cases result in lowered sensitivity (see tables S5−S6).

Human genetic background				*Epipedobates* genetic background			
Genotype	**AA replacement(s)**	**Fold change in EC_{50} ACh**	**Fold change in EC_{50} epibatidine**	**Genotype**	**AA replacement(s)**	**Fold change in EC_{50} ACh**	**Fold change in EC_{50} epibatidine**
FSAI	(wild-type)	reference	reference	**FS**AI	L106F C108S	reference	reference
LCAI	F106L S108C	1	17**	LCAI	(wild-type)	1	44**
LSAI	F106L	1	1	L**S**AI	C108S	1	1
F**C**AI	S108C	>1	49**	**F**CAI	L106F	2*	138**
F**CVV**	S108C A110V I118V	1	6**	–	–	–	–
FS**V**I	A110V	1	1	–	–	–	–
FSA**V**	I118V	>1	75**	–	–	–	–

*, $p < 0.01$;

**, $p < 0.001$ (two-way ANOVA, corrected for multiple comparisons using Tukey's test; see tables S9, S11, S13, and S14).