

Molecular interaction of α -conotoxin RgIA with the rat $\alpha 9\alpha 10$ nAChR

Layla Azam, Athanasios Papakyriakou, Marios Zouridakis, Petros Giastas, Socrates J. Tzartos and J. Michael McIntosh

Departments of Biology (LA, JMM) and Psychiatry (JMM), University of Utah, Salt Lake City, UT and George E. Wahlen Veterans Affairs Medical Center (JMM), Salt Lake City, UT., National Center for Scientific Research “Demokritos” (AP), Athens, Greece and Department of Neurobiology (MZ, PG, SJT), Hellenic Pasteur Institute, Athens, Greece.

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Corresponding author: Layla Azam, University of Utah, Dept. of Biology, 257 South 1400 East, Salt Lake City, UT 84112. Telephone: 801-5818370; Fax: 801-581-5010.
layla.azam@utah.edu

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Abbreviations: nAChR, nicotinic acetylcholine receptor; ECD, extracellular domain; α -CTx, α -conotoxin; ACh, acetylcholine; AChBP, acetylcholine-binding protein; BSA, bovine serum albumin; MD, molecular dynamics

Abstract

The $\alpha 9\alpha 10$ nicotinic acetylcholine receptor (nAChR) was first identified in the auditory system, where it mediates synaptic transmission between efferent olivocochlear cholinergic fibers and cochlea hair cells. This receptor gained further attention due to its potential role in chronic pain and breast and lung cancers. We previously showed that α -conotoxin (CTx) RgIA, one of the few $\alpha 9\alpha 10$ selective ligands identified to date, is 300-fold less potent on human vs. rat $\alpha 9\alpha 10$ nAChR. This species difference was conferred by only one residue in the (-), rather than (+), binding region of the $\alpha 9$ subunit. In light of this unexpected discovery, we sought to determine other interacting residues with α -CTx RgIA. A previous molecular modeling study, based on the structure of the homologous molluscan acetylcholine-binding protein, predicted that RgIA interacts with three residues on the $\alpha 9(+)$ face and two residues on the $\alpha 10(-)$ face of the $\alpha 9\alpha 10$ nAChR. However, mutations of these residues had little or no effect on toxin block of the $\alpha 9\alpha 10$ nAChR. In contrast, mutations of homologous residues in the opposing nAChR subunits ($\alpha 10$ E197, P200 and $\alpha 9$ T61, D121) resulted in 19- to 1700-fold loss of toxin activity. Based on the crystal structure of the extracellular domain (ECD) of human $\alpha 9$ nAChR, we modeled the rat $\alpha 9\alpha 10$ ECD and its complexes with α -CTx RgIA and acetylcholine (ACh). Our data support the interaction of α -CTx RgIA at the $\alpha 10/\alpha 9$ rather than the $\alpha 9/\alpha 10$ nAChR subunit interface, and may facilitate the development of selective ligands with therapeutic potential.

Introduction

nAChRs are members of the Cys-loop family of ligand gated ion channels, whose other members include 5HT₃, the GABA_A and glycine receptors (Bouzat, 2012; Lester et al., 2004; Sine and Engel, 2006). nAChRs are pentameric receptors found both at the neuromuscular junction as well as in central and peripheral neurons. The neuronal nAChRs are composed of combination of α and β subunits. So far, nine non-muscle α (α 2- α 10) and three β (β 2- β 4) subunits have been identified (Albuquerque et al., 2009). Different combinations of α/β (heteromeric) or α/α subunits (homomeric) result in formation of nAChRs with distinct pharmacology and localization within both the central and peripheral nervous system (Gotti et al., 2007; Gotti et al., 2006; Millar and Gotti, 2009). One of these subunits, α 9, was initially localized in cochlear inner and outer hair cells (Elgoyhen et al., 1994). Although originally thought to form a homomeric α 9 nAChR, subsequent studies identified the α 10 subunit as a partner (Elgoyhen et al., 2001; Lustig et al., 2001; Sgard et al., 2002). Although similar to homomeric α 9 nAChRs, heteromeric α 9 α 10 nAChRs more closely resemble the pharmacological and electrophysiological properties of native nAChRs found in cochlear hair cells (Elgoyhen et al., 2001; Lustig, 2006; Sgard et al., 2002). In addition, both knockout and developmental studies indicate that responses of cochlear hair cells to ACh are mediated through α 9 α 10 nAChRs (Katz et al., 2004; Roux et al., 2011; Vetter et al., 2007; Vetter et al., 1999).

In addition to the auditory system, transcripts and/or protein for α 9 and α 10 subunits have been found in lymphocytes, skin keratinocytes, sperm, dorsal root ganglion, sympathetic neurons, macrophages, and adrenal chromaffin cells (Colomer et

al., 2010; Haberberger et al., 2004; Kumar and Meizel, 2005; Kurzen et al., 2004; Lips et al., 2002; Lustig et al., 2001; Nguyen et al., 2000; Peng et al., 2004; Simard et al., 2013).

The physiological role of $\alpha 9\alpha 10$ nAChRs in the auditory system has been determined through studies done with $\alpha 9$ and $\alpha 10$ knockout mice (Vetter et al., 2007; Vetter et al., 1999). These studies suggest an important role for both subunits in the development of cochlear morphology and innervation, as well as in the regulation of normal suppression of cochlear responses after olivocochlear fiber stimulation (Vetter et al., 2007; Vetter et al., 1999).

In contrast to the auditory system, the physiological role of $\alpha 9\alpha 10$ nAChRs in other areas has been more difficult to assess. For example, in human lymphocytes that co-express mRNA for both $\alpha 9$ and $\alpha 10$ subunits, ACh failed to generate an ionic current, suggesting a physiological role of $\alpha 9\alpha 10$ nAChRs different from that observed in cochlear hair cells (Peng et al., 2004). Recently, it was shown that α -CTx RgIA, a specific blocker of $\alpha 9\alpha 10$ nAChRs (Ellison et al., 2006) is analgesic in a rat model of nerve injury (Vincler et al., 2006). This analgesic effect was attributed to a decrease in the number of immune cells recruited to the site of injury (Vincler et al., 2006).

The potency of α -CTx RgIA for heterologously expressed rat vs. native $\alpha 9\alpha 10$ nAChRs expressed in hair cells matches well (Ellison et al., 2006). However, upon comparing the potency of α -CTx RgIA on heterologously expressed rat vs. human $\alpha 9\alpha 10$ nAChRs, we observed a >300 fold difference in potency between the two species, with the toxin being less potent on the human subtype. Subsequent mutation studies indicated that this difference is conferred by a single amino acid in the (-) binding region of the $\alpha 9$ subunit (Azam and McIntosh, 2012). In the current study, we have performed

an in-depth structure/function analysis of the interaction of α -CTx RgIA with the $\alpha 9\alpha 10$ nAChR.

Materials and Methods

Acetylcholine chloride, atropine, and bovine serum albumin (BSA) were obtained from Sigma. α -CTxs were synthesized as described previously (Ellison et al., 2006; McIntosh et al., 2005). Clones of rat $\alpha 9$ and $\alpha 10$ cDNAs in pGEMHe and pSGEM vectors, respectively, were kindly provided by A. Belen Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina).

Construction of point mutations- Point mutants were made by PCR. Primers containing the desired point mutation flanked by at least 15 bases on either side were synthesized. Using the non-strand displacing action of *Pfu Turbo* DNA polymerase, mutagenic primers were extended and incorporated by PCR. The methylated, nonmutated parental cDNA was digested with *Dpn I*. Mutated DNA was transformed into DH10B or DH5 α competent cells and isolated using the Qiagen miniprep kit and sequenced to ascertain the incorporation of the desired mutation.

cRNA preparation and injection- Capped cRNA for the various subunits were made using the mMessage mMachine *in vitro* transcription kit (Ambion, Austin, TX) following linearization of the plasmid. cRNA was purified using a Qiagen RNeasy kit (Qiagen, Valencia, CA). The concentration of cRNA was determined by absorbance at 260 nm. cRNA of either wildtype or mutant subunits were mixed at a 1:1 ratio with wildtype subunits for a final concentration of at least 500 ng/ μ l for each subunit cRNA. One hundred to 150 nl of this mixture was injected into each *Xenopus* oocyte with a

Drummond microdispenser (Drummond Scientific, Broomall, PA), as described previously (Cartier et al., 1996), and incubated at 17°C. Oocytes were injected within one day of harvesting and recordings were made 2-4 days post-injection.

Voltage-clamp Recording-For the antagonist dose response experiments, oocytes were voltage-clamped and exposed to ACh and peptide as described previously (Cartier et al., 1996). Briefly, the oocyte chamber consisting of a cylindrical well (~30 μ l in volume) was gravity perfused at a rate of ~2 ml/min with ND-96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.1-7.5, supplemented with 0.1 mg/ml BSA). In the experiments with Ca²⁺-free ND96, 1.8 mM BaCl was substituted. Oocytes were subjected once a minute to a 1 sec pulse of 100 μ M ACh. For toxin concentrations of 1 μ M and lower, once a stable baseline was achieved, either ND-96 alone or ND-96 containing varying concentrations of the α -CTxs were perfusion-applied, during which 1-sec pulses of 100 μ M ACh (200 μ M for α 7 nAChR) were applied every 90 sec until a constant level of block was achieved. For toxin concentrations of 10 μ M and higher, the buffer flow was stopped and the toxin bath-applied and allowed to incubate with the oocyte for 5 min, after which the ACh pulse was resumed.

To acquire ACh dose-response data, the conventional oocyte chamber was replaced by a chamber constructed from a disposable 200 μ l polypropylene pipette tip with a length of 50 mm and an internal diameter of 0.5 mm at the upstream or intake end and 5 mm at the downstream or exhaust end. The chamber was mounted horizontally with its intake end connected to the perfusion supply, while its exhaust end had a vertical meniscus whose location was dictated by the tip of a sipper made from a 27 gauge

hypodermic needle connected to a vacuum line. The chamber had two apertures in its dorsal wall: 1) a 1.5 mm circular hole centered 13 mm downstream from the intake, and 2) a 2.5 x 5 mm (longitudinal) oval centered 14 mm downstream from the hole (i.e., a total of 27 mm from the intake end). Oocytes were introduced into the chamber through the oval aperture and secured against the chamber floor by two voltage-clamp glass microelectrodes that impaled the oocyte. The chamber was perfused at a rate of ~ 1 ml/min. To introduce ACh into the chamber, the perfusion was halted and 20 μ l of ACh was manually applied to the chamber via the small circular hole upstream from the oocyte. This volume was too small for ACh to reach the oocyte unless the perfusion was resumed. Upon resumption of perfusion (which was started immediately following the introduction of ACh into the chamber), the bolus of ACh rapidly engulfed the oocyte and washed past it in a matter of seconds, as judged by the time course of ACh response. This process was repeated with different concentrations of ACh with a time interval between applications long enough to avoid desensitization.

Data Analysis-For the baseline measurement, at least three ACh responses were averaged. To determine the percent block induced by toxin, 2-3 ACh responses, obtained after a steady state block had been achieved, were averaged and the value divided by the pre-toxin baseline value to yield a % response. Dose-response data were fit to the equation, $Y = 100 / (1 + 10^{((\text{LogEC}_{50} - \text{Log}[\text{Toxin}]) \times n_H))}$, where n_H is the Hill coefficient, by non-linear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). Each data point is the mean \pm SEM from at least 3 oocytes. For ACh dose-response curves, the response to a given ACh concentration was normalized to the response to 100 μ M ACh, which served as an internal control.

Molecular modeling methods-The molecular model of the ECD of the rat $(\alpha 9)_2(\alpha 10)_3$ AChR was based on the high-resolution (1.7 Å) X-ray crystal structure of the monomeric state of the ECD of the human $\alpha 9$ nAChR in its complex with the antagonist methyllycaconitine (PDB ID: 4UXU) (Zouridakis et al 2014). All non-protein atoms and the alternative location B of residues H63 and N109 with the lowest occupancy were removed from the template structure. The sequence alignment between the human and rat ECDs (Supplementary Figure S1) was performed with ClustalW2 using the UNIPROT accession codes P43144 for rat $\alpha 9$ (96.2 % sequence identity for 212 residues) and Q9JLB5 for rat $\alpha 10$ (66.7 % sequence identity). The homology models of the rat $\alpha 9$ and $\alpha 10$ monomers were prepared using Modeller v9.10 (Fiser and Sali, 2003) and the best models were selected on the basis of the lowest DOPE score amongst 30 models generated. The initial model of the α -CTx RgIA was taken from the representative conformation (model 1) of its NMR structure (PDB ID: 2JUQ) (Ellison et al. 2008). The $(\alpha 9)_2(\alpha 10)_3$ ECD was prepared by superimposing each of the two rat monomeric models on the crystallographic structure of the *Aplysia californica* acetylcholine-binding protein (AChBP) complex with α -CTx ImI (PDB ID: 2BYP) (Hansen et al., 2005) using the MULTISEQ plugin of VMD v1.9 (Humphrey et al., 1996). Specifically, $\alpha 10$ ECD was superimposed with chains A, B and D, whereas $\alpha 9$ ECD with chains C and E, so that two $\alpha 10(+)\alpha 9(-)$ binding sites between chains B, C and D, E were generated. The final model of the rat $(\alpha 9)_2(\alpha 10)_3$ complex with α -CTx RgIA was prepared by superimposing the NMR structure of RgIA with chains F and I of ImI in the AChBP complex. The $\alpha 9\alpha 10P200Q$ mutant was prepared by changing P200 of a single $\alpha 10$ subunit to glutamine in the final model. The model of the rat $(\alpha 9)_2(\alpha 10)_3$ complex with ACh was

prepared by a similar procedure based on the X-ray crystal structure of the *Lymnaea stagnalis* AChBP complex with carbamylcholine (PDB ID: 1UV6) (Celie et al., 2004). Ligand molecules were placed at the five ligand-binding sites by changing only the amide nitrogen atom of carbamylcholine to carbon and then adding hydrogen atoms.

Molecular dynamics (MD) simulations- MD simulations were performed using the GPU-PMEMD program of AMBER v12 software (Case et al., 2005; Salomon-Ferrer et al., 2013). The ff12SB force field parameters (Hornak et al., 2006) were employed for the receptor and the α -CTx RgIA, while parameters from the GAFF (Wang et al., 2004) with AM1-BCC charges were applied to the ligands using the LEaP module. The systems were solvated in truncated octahedron boxes of TIP3P waters with a minimum extension of 12 Å from the solute, and the total charge was neutralized with the addition of sodium ions. Simulations were performed with a 2-fs time step and the SHAKE algorithm to constrain all bonds involving hydrogen atoms. The Particle Mesh Ewald method was used for long-range electrostatic interactions and an 8-Å cutoff radius was used for range-limited interactions. Initially, the system was minimized and then the temperature increased gradually from 10 K to 300 K within 100 ps using harmonic positional restraints of 10 kcal/mol/Å² on the protein and peptide backbone atoms. The restraints were then gradually removed within 200 ps and the system was equilibrated for 10 ns at constant isotropic pressure of 1 atm and temperature of 300 K, using the Berendsen control algorithms. The production simulation was carried out for 100 ns in the isothermal-isobaric (NPT) ensemble with a pressure relaxation time of 2 ps, using a Langevin thermostat at 300 K with a collision frequency of 2 ps. Snapshots were collected every 2 ps and were processed using the PTRAJ module of AMBER. The

structures were clustered using a hierarchical agglomerative approach with a minimum distance between clusters of 1.5 Å, after mass-weighted, root-mean-square deviation fitting of all C^α atoms (Data Supplement 1 and 2). Calculations were performed on an Intel workstation equipped with NVIDIA GTX 780 GPUs running a Linux 2.6.32 86_x64 kernel.

Results

Identification of determinants of α -CTx RgIA interaction with α 9 and α 10 subunit residues

Competitive nicotinic ligands bind to the interface of adjacent nAChR subunits. The α 9 α 10 nAChR may have two or more ligand binding interfaces that lie at the junction between α 9 and α 10 subunits. These binding interfaces have historically been believed to occur between the (+) side of α 9 and the (-) face of α 10, with the α 10 being considered the “structural” subunit (Plazas et al., 2005). Part of the rationale for this thinking is that since the α 9 subunit can assemble into a functional homomer, ACh must bind near the C-loop located on the (+) face of the α 9 subunit. Based on these assumptions, Perez et al., used molecular modeling to predict binding interactions of α -CTx RgIA with the α 9 α 10 nAChR. The crystal structure of the AChBP bound to α -CTx ImI was used as a template to build these 3D models. MD simulations suggested that α -CTx RgIA interacts with residues on the (+) face of α 9 and the (-) face of α 10 subunits (Perez et al., 2009). This study specifically predicted interaction of α -CTx RgIA R11 and R7 with E194 and with D198 and P197, respectively, in the α 9 nAChR subunit,

located on the (+) or principal face of the nAChR. The study further predicted interaction of α -CTx RgIA Arg9 with E58 and D114 in the α 10 subunit, located on the (-) or complementary binding site. In order to comply with the numbering of the α 9 nAChR subunit presented in the recent crystal structure of the mature human α 9 ECD (Zouridakis et al 2014), the α 9-interacting residues with α -CTx RgIA suggested by Perez et al. are E197, D201 and P200 in this study, while the α 10-interacting residues are E61 and D121 (Fig. 1A).

Based on predictions by the Perez et al., study we mutated residues in the rat α 9(+) and α 10(-) binding sites. α 9E197Q α 10, α 9P200Q α 10 and α 9D201N α 10 were tested for changes in α -CTx RgIA sensitivity. Surprisingly, none of these mutations in the α 9(+) binding site had any effect on α -CTx RgIA potency. The potency of α -CTx RgIA on all of these mutants was similar to the wild-type α 9 α 10 nAChR (Table 1, Fig. 2A).

In the (-) face of α 10 subunit, E61 was changed to Ile and Thr and D121 to Leu. None of these mutations markedly affected the potency of α -CTx RgIA (Table 2, Fig. 2B). Thus, neither the α 9 nor the α 10 receptor residues predicted by Perez et al., to interact with α -CTx RgIA appear to substantially influence binding.

Interestingly, E61 in the α 10 subunit, which Perez et al., suggested to interact with α -CTx RgIA (E58 in their study), corresponds to T61 in the α 9 subunit, which in our previous study was shown to contribute to high potency of α -CTx RgIA on the rat vs. human α 9 α 10 nAChR (Azam and McIntosh, 2012). This implied that the α -CTx RgIA interaction might occur at an α 10/ α 9 rather than an α 9/ α 10 subunit interface. To test the

possibility that the (-) face residues in the $\alpha 9$ and (+) face residues in the $\alpha 10$ subunit interact with α -CTx RgIA (opposite of that suggested by Perez et al.), we mutated the $\alpha 10(+)$ face and the $\alpha 9(-)$ face residues at the respective homologous positions of the $\alpha 9(+)$ face and $\alpha 10(-)$ face.

Each mutation of the respective $\alpha 9(-)$ face residues caused a loss in α -CTx RgIA potency. More specifically, when $\alpha 9D121$ was mutated to a Leu residue, found in the corresponding region of the $\alpha 7$ subunit which has low sensitivity to α -CTx RgIA (IC_{50} of 7.27 μM ; 95% CI 6.13-8.62 μM , Fig. 3), the activity of α -CTx RgIA was nearly abolished on $\alpha 9D121L\alpha 10$; more precisely, no activity was shown at α -CTx RgIA concentrations as high as 1 μM (response after 1 μM α -CTx RgIA: $101 \pm 5.7\%$ of control) (Table 1, Fig. 2A). However, when the converse mutation was made in the $\alpha 7$ subunit, where $\alpha 7L119$ (corresponds to $\alpha 9D121$) was replaced with an Asp, the $\alpha 7L119D$ receptor gained more than 10-fold sensitivity to α -CTx RgIA, with an IC_{50} of 536 nM (95% CI 414-693nM; Fig. 3). Interestingly, the $\alpha 7S59T$ ($\alpha 7S59$ corresponds to rat $\alpha 9T61$), did not change sensitivity towards α -CTx RgIA (Fig. 3) suggesting that Thr in this position is not imperative for α -CTx RgIA binding to all subunits. Concerning the rat $\alpha 9T61$ residue, since we had previously shown that its replacement with an Ile residue, found in the homologous position in the human $\alpha 9$ subunit, dramatically affected α -CTx RgIA potency (Azam and McIntosh, 2012) (Table 1), we now sought to determine whether mutation of this Thr residue to something other than a Ile still had an effect on α -CTx RgIA activity. We chose Glu, as this residue is found in the homologous position in the $\alpha 10$ subunit and was predicted to interact with R9 of α -CTx RgIA (Perez et al.,

2009). $\alpha 9\text{T61E}$ had an almost 20-fold lower sensitivity to $\alpha\text{-CTx RgIA}$ compared to wild-type receptor (Table 1).

Likewise, when the (+) face residues of the $\alpha 10$ subunit were mutated, the potency of $\alpha\text{-CTx RgIA}$ was affected. $\alpha 9\alpha 10\text{E197Q}$ and $\alpha 9\alpha 10\text{P200Q}$ were 25- and 300-fold respectively, less sensitive to $\alpha\text{-CTx RgIA}$ compared to wild-type $\alpha 9\alpha 10$ nAChR (Table 2; Fig. 2B). Unfortunately, the $\alpha 10\text{D201N}$ mutation did not consistently yield functional expression and therefore was not tested.

Previous studies in *Xenopus* oocytes have indicated that Ca^{2+} entry (such as through opening of $\alpha 9\alpha 10$ nAChRs) can lead to activation of Ca^{2+} -dependent Cl^- channels (Barish, 1983). At the holding potential used in this study (-70 mV) this can cause a large negative current, in addition to the inward positive current through the $\alpha 9\alpha 10$ nAChRs. Therefore, we performed a set of electrophysiological experiments using a buffer where Ba^{2+} was used in place of Ca^{2+} to exclude the possibility that block by the toxin was mediated by block of Ca^{2+} -dependent Cl^- channels rather than block of $\alpha 9\alpha 10$ nAChRs. In Ba^{2+} buffer, measured currents were $\leq 10\%$ of those observed in the Ca^{2+} buffer (unpublished observation). However, the potency of $\alpha\text{-CTx RgIA}$ for blocking the $\alpha 9\alpha 10$ nAChR in the Ba^{2+} (2.05 nM; 95% CI 1.48-2.85 nM) did not significantly differ from that observed in regular ND-96 buffer (1.49 nM; 95% CI 1.13-1.95 nM). Unfortunately, when most of the receptors formed by the mutant $\alpha 9$ or $\alpha 10$ subunits were tested in the Ba^{2+} buffer, the currents obtained were too small for accurate measurement of $\alpha\text{-CTx RgIA}$ potency. Only one of the mutants, $\alpha 9\text{T61I}\alpha 10$ gave robust currents in the Ba^{2+} -containing buffer. The IC_{50} for block of $\alpha\text{-CTx RgIA}$

was similar in Ca²⁺-containing (2.61 μ M, Table 1) and Ca²⁺-free (4.93 μ M, 95% CI 2.4-11.4 μ M) buffers.

Effect of mutations in the α 9 and α 10 subunits affecting RgIA on ACh sensitivity

The mutations in both the α 9 and the α 10 subunits that affected α -CTx RgIA potency were tested for any changes in ACh sensitivity. ACh activated wild-type rat α 9 α 10 nAChR with an EC₅₀ value of 10.1 μ M (Table 3). The mutated (+) face α 10 residues E197 and P200 caused an approximately 20-fold (mutant α 10E197Q) and a 40-fold rightward shift (mutant α 10P200Q) in ACh potency, respectively (Table 3, Fig. 4). The mutated (-) face α 9 residues had differential effects on ACh potency. The α 9T61I mutant that caused a >1,500-fold change in α -CTx RgIA potency (see above), did not affect ACh potency. However, α 9D121L α 10 lowered the ACh potency by about 30-fold (Table 3; Fig. 4).

Molecular modeling of the rat α 9 α 10 ECD based on the X-ray crystal structure of the human α 9 ECD

Molecular modeling studies of the complex of α 9 α 10 ECD with α -CTx RgIA

To gain insight into the molecular basis of the interaction between the rat nAChR and α -CTx RgIA, we employed MD calculations of the ECD of the rat (α 9)₂(α 10)₃ nAChR complex with α -CTx RgIA. Our model was based on the recent X-ray crystal structure of the monomeric state of the ECD of the human α 9 nAChR (Fig. 5A) (Zouridakis et al. 2014) and the solution NMR structure of α -CTx RgIA (Fig. 5B) (Ellison et al. 2008). Molecular models of the highly homologous rat α 9 and α 10 subunits

were assembled in the pentameric state (Fig. 5C, D) on the basis of the AChBP complex with α -CTx ImI as described in the Methods section. Two α -CTx molecules were modeled in the two α 10(+)/ α 9(-) binding sites (Fig. 5C) and unrestrained MD simulations in explicit solvent were carried out (Data Supplement 1). The systems exhibited significant stability during the course of the MD simulations as demonstrated by the root-mean-square deviations from the initial conformation and the radius of gyration of the receptor (Supplementary Figs. S2 and S3). The bound α -CTx molecules displayed similar arrangement within the two α 10/ α 9 sites but did not exhibit identical interactions as a function of simulation time, especially with respect to the C-terminal R13 (see below).

At the α 10(+) side (Fig. 6A), E197 displayed a quite stable electrostatic interaction with the side chain of R11 (Figs. 6B, C). Therefore, the significant decrease in the sensitivity to α -CTx RgIA for the mutant α 9 α 10E197Q (Table 2) could be due to the impairment of the formation of a salt bridge with R11. In addition, the carbonyl group of P200 formed a stable hydrogen bond with the guanidinium group of R7, which was stabilized by an electrostatic interaction between D201 and R7 (Fig. 6B). Since the predicted interaction of R7 with the main chain of P200 cannot readily explain the observed reduction in the potency of α -CTx RgIA by 300 times for the α 9 α 10P200Q mutant, we carried out MD of the same model, but with a single P200Q mutation at one of the two α 10 α 9 sites. In this case, our calculations revealed that the guanidinium group of R7 and the backbone C=O group of Q200 were not within hydrogen-bonding distance throughout the course of the MD simulations (Fig. 6D). This can be justified by the higher flexibility of Gln with respect to Pro, which allows a rotation of the backbone carbonyl group away from R7. Although we were not able to obtain functional expression

for the $\alpha 10D201N$ mutation, our modeling data indicated that the interaction between R7 and $\alpha 10D201$ was preserved during the MD simulations (Supplementary Figure S4), which suggests the importance of an Asp residue in this position for binding of α -CTx RgIA.

At the $\alpha 9(-)$ face, D121 formed a stable salt bridge interaction with R9 of α -CTx RgIA throughout the MD simulations in both $\alpha 10\alpha 9$ sites (Figs. 7A, B, C), whose importance was depicted at the >670-fold decrease in the potency of α -CTx RgIA in the $\alpha 9D121L\alpha 10$ mutant (Table 1). In one $\alpha 10/\alpha 9$ site, D121 also exhibited a stable hydrogen-bonding intramolecular interaction with R59, which was also apparent at the X-ray crystal structure of the monomeric human $\alpha 9$ ECD (Zouridakis et al. 2014). At this site, our simulations did not reveal any direct interaction between the antagonist and T61, rather than water-mediated interactions between the $\alpha 9$ T61 hydroxyl group and the backbone of the R9–Y10–R11 moiety. At the other $\alpha 10/\alpha 9$ binding site, R59 shifted away from D121 and formed a salt bridge interaction with R13 of α -CTx RgIA (Figs. 7B, D). This movement had no effect in the intermolecular interaction between $\alpha 9$ D121 and R9 of α -CTx RgIA; however, the side chain of R13 displayed hydrogen bonding interactions with the hydroxyl group of $\alpha 9$ T61, albeit for a fraction of the simulation time as a result of the high mobility of R13 (Figs. 7B, E). Therefore, the significant effect of the $\alpha 9T61I\alpha 10$ mutant in the potency of α -CTx RgIA (Table 3, Fig. 2) could be attributed to the loss of a potent hydrogen bond with R13 of α -CTx RgIA and/or the altered hydration degree of the $\alpha 9(-)$ site.

Nicotinic receptors are members of the Cys-loop ligand-gated ion channel superfamily. Other evolutionarily related members include GABA_A, glycine and 5-HT₃

receptors. Each of these are formed from five subunits arranged around a central ion-conducting pore; each subunit has a characteristic “Cys-loop” formed by two highly conserved Cys residues of the ECD. We aligned the ECDs of these receptors to examine possible homology with the critical residues identified for the $\alpha 9$ and $\alpha 10$ nAChRs. E197 was conserved in the 5HT3 receptor, but the other residues were not conserved among the non-nicotinic receptors (Supplementary Figure S5). This is consistent with α -CTx RgIA having an $IC_{50} > 10 \mu M$ for the 5-HT3 and GABA_A receptor in competitive binding assays (unpublished results), in contrast to potent block of $\alpha 9\alpha 10$ nAChRs.

Molecular modeling studies of the complex of $\alpha 9\alpha 10$ ECD with ACh

In the molecular model of the rat $(\alpha 9)_2(\alpha 10)_3$ nAChR complex with ACh, the agonist is surrounded by an aromatic cage comprising Y95, Y192, Y199 and W151 from the $\alpha 10(+)$ subunit and displays additional interactions with the C194–C195 disulfide bridge, S150 and E197 (Fig. 8; Data Supplement 2). Interestingly, $\alpha 10E197$ formed a salt bridge with $\alpha 9R81$; therefore it is possible that the $\alpha 10E197Q$ mutation disrupted the optimum conformation of the $\alpha 10/\alpha 9$ binding site, as demonstrated by the 20-fold increase in the EC_{50} value for ACh (Table 3). Similarly, it is possible that the $\alpha 10P200Q$ mutation resulted in local conformational rearrangements that affected the interaction of ACh with the neighboring Y199 residue by 40-fold, respectively (Table 3). At the $\alpha 9(-)$ side, the carboxylate group of D121 is predicted to form electrostatic interactions with the trimethyl ammonium moiety of ACh, the impairment of which led to a 30-fold increase of the EC_{50} value in the $\alpha 9D121L\alpha 10$ mutant (Table 3). In addition, V111 and the aliphatic moiety of R81 formed van der Waals interactions with the acetoxy group of ACh (Fig. 8).

Discussion

In this study, we demonstrated asymmetrical interaction of α -CTx RgIA with the rat $\alpha 9\alpha 10$ nAChR. The mutational studies indicated interaction of α -CTx RgIA with the $\alpha 10(+)/\alpha 9(-)$ interface of the $\alpha 9\alpha 10$ nAChR, which is opposite to that proposed by previous modeling studies based on the structure of the homologous AChBP from invertebrates (Perez et al., 2009). Interestingly, however, the residues of interaction of the $\alpha 10(+)$ and of the $\alpha 9(-)$ faces found in our study are in homologous positions to those predicted by Perez et al. for the opposite subunits. Thus, the Perez et al., model though incorrect in assignment of principal and complementary binding site subunits, succeeded in assigning interacting residues if subunit interface is not considered. Based on the recent X-ray crystal structure of the ECD of human $\alpha 9$ nAChR, we performed MD simulations of the rat $\alpha 9\alpha 10$ ECD complexes with α -CTx RgIA to provide a molecular basis for the results of our functional studies. The current model is probably more accurate than the previous ones based on the homologous AChBPs, since the sequence identities of rat $\alpha 9$ and $\alpha 10$ ECDs with human $\alpha 9$ ECD are 96% and 67%, respectively, whereas the identities with AChBP are below 25%. Indeed, the results of these simulations were consistent with our functional studies.

The muscle nAChR was the first nicotinic receptor to be rigorously examined with respect to selective toxin binding to subunit interface. Two ACh binding sites are formed between its $\alpha 1/\delta$ and $\alpha 1/\gamma$ (or ϵ in adult) interfaces. Sine and co-workers demonstrated that a paralytic peptide, α -CTx MI, selectively binds the $\alpha 1/\delta$ interface (Sine et al., 1995; Sugiyama et al., 1998); subsequently other toxins were discovered that

selectively bind the $\alpha 1/\delta$, $\alpha 1/\epsilon$ or $\alpha 1/\gamma$ interfaces (Groebe et al., 1995; Hann et al., 1994; Martinez et al., 1995; Molles et al., 2002a; Molles et al., 2002b), which allowed the development of radiolabeled and fluorescent probes for specific labeling of fetal or adult muscle nAChR subtypes (Teichert et al., 2008).

Neuronal nAChRs are composed of α and β subunits. Their ligand-binding sites have been shown to be formed at the interfaces between the (+) face of an α subunit and the (-) face of a β subunit; several conotoxins have been discovered that discriminate between the different subunit interfaces. α -CTx MII (Cartier et al., 1996), for example, shows high discrimination between $\alpha 6/\beta 2$ and $\alpha 4/\beta 2$ subunit interfaces (Salminen et al., 2004). Thus, α -CTxs have greatly contributed to the molecular dissection of neuronal nAChR subtypes that modulate the release of neurotransmitters. The present study shows that α -CTx RgIA can discriminate among asymmetrical heteromeric α/α subunit interfaces; in the rat $\alpha 9\alpha 10$ nAChR, α -CTx RgIA selectively interacts with $\alpha 10(+)/\alpha 9(-)$ vs. $\alpha 9(+)/\alpha 10(-)$ interfaces. The $\alpha 9$ subunit, in contrast to the $\alpha 10$, forms homopentamers and that is one reason it was originally assumed that competitive ligands would bind to the (+) face of the $\alpha 9$ subunit and (-) face of either $\alpha 9$ (homomeric nAChR) or (-) face of $\alpha 10$ in the heteromeric $\alpha 9\alpha 10$ nAChR. In support of this, Ellison et al., showed that mutation of W176 (W151 based on numbering in this study) to Thr in the $\alpha 9(+)$ face resulted in loss of potency for ACh and α -CTx RgIA, consistent with competitive interaction of α -CTx RgIA at an agonist binding site (Ellison et al., 2008). In addition, crystallization of the ECD of the human $\alpha 9$ subunit in the presence of antagonists indicated a major contribution of the $\alpha 9(+)$ face for binding of

methyllycaconitine and α -bungarotoxin (Zouridakis et al., 2014). However, the present study showed that there is a separate, high affinity, binding site for α -CTx RgIA and ACh, which is the α 10(+)/ α 9(-) interface.

Based on the model of rat α 9 α 10 nAChR suggested by Perez et al., we mutated the α 9(+) and α 10(-) face residues shown to interact with α -CTx RgIA and investigated their effect. The α 9(+) face residues proposed to interact with R11 and R7 of the toxin were E194 and P197/D198, respectively. When any of these residues was mutated in the α 9 subunit (E197, P200 and D201 in this study), there was no effect on α -CTx RgIA potency (Table 1). In contrast, when the same residues in the α 10(+) face were mutated (α 10 E197 and P200Q) there was a 25-300 fold loss in toxin activity (Table 2). This effect is much larger than the 5-fold reduction in potency for the W176T mutation in the α 9(+) face found in the Ellison et al., study (W151T based on our numbering). In addition, both the E197Q and P200Q mutations in the α 10(+) face that affect α -CTx RgIA binding also decrease the potency of ACh for activation of the α 9 α 10 nAChR (Table 3).

Our molecular modeling studies suggested putative interactions between α 10E197 and R11 of α -CTx RgIA, as well as between α 10P200, α 10D201 and R7 of α -CTx RgIA in the α 10(+)/ α 9(-) binding interface (Fig. 6), in support to our mutational studies. Interestingly, in all other α subunits, conferring the (+) faces of ACh-binding sites, with the exception of α 5 (which has not been reported to participate in ACh binding sites), a negatively charged residue exists in the homologous positions to α 10 E197 and D201 (Fig. 1B). P200, whose main chain is predicted to interact with α -CTx RgIA, is also highly conserved, with the exception of α 1 and α 6, in which a Tyr or a Thr residue exists

at this position, respectively (Fig. 1B). This Thr in the $\alpha 6$ subunit has been shown to confer high potency of an α -CTx MII analog on the $\alpha 6\beta 2\beta 3$ nAChR (Azam et al., 2008).

In addition, Perez et al. also suggested a role for the $\alpha 10(-)$ face residues E61 and D121 (our numbering) in interacting with α -CTx RgIA (Perez et al., 2009). However, we did not observe a dramatic change in α -CTx RgIA potency when either residue in the $\alpha 10$ subunit was mutated to an Ile/Thr or Leu, respectively (Table 2). Instead, D121L mutation in the $\alpha 9(-)$ face caused a complete loss of sensitivity to α -CTx RgIA (Table 1). We chose Leu as a substitute for Asp, because this residue exists in the homologous position of the $\alpha 7$ subunit ($\alpha 7$ L119), which has low sensitivity to α -CTx RgIA (Ellison et al., 2006). Consistent with this, when the converse mutation was made in the $\alpha 7$ subunit, the homopentamer formed from the mutant subunit $\alpha 7$ L119D had >10-fold higher sensitivity to α -CTx RgIA. According to our model, D121 of the $\alpha 9(-)$ face forms a salt bridge with R9 of α -CTx RgIA, similar to the D121 of the $\alpha 10(-)$ face in the Perez et al. study (Perez et al., 2009). Notably, among nAChR subunits, this negatively charged residue is only found in the $\alpha 9$ and $\alpha 10$ subunits (Figure 1B), suggesting that $\alpha 9$ D121 is critical for activity and selectivity of α -CTx RgIA for the $\alpha 9\alpha 10$ nAChR.

In position 61 of the rat $\alpha 9$ subunit a Thr residue exists instead of Glu, which is present in $\alpha 10$ (Fig. 1A). A Glu residue in this position also exists in the $(-)$ face of the binding sites of $\beta 2$ and $\beta 4$ subunits. $\alpha 9$ T61 was previously shown (Azam and McIntosh, 2012) to confer ~300-fold higher potency of α -CTx RgIA on the rat vs. human $\alpha 9$ subunit, which instead has an Ile residue at this position (Fig. 1B). In addition, when we mutated $\alpha 9$ T61 to Glu (found in $\alpha 10$, $\beta 2$, $\beta 3$ and $\beta 4$) in the present study, a ~20-fold

decrease in the potency for α -CTx RgIA was observed (Table 1), confirming that $\alpha 9$ confers the (-) face of α -CTx binding site in the $\alpha 9\alpha 10$ nAChR. Our MD simulations of the rat $\alpha 10(+)/\alpha 9(-)$ binding interface displayed water-mediated interactions of $\alpha 9T61$ with the R9-Y10-R11 moiety of α -CTx RgIA, as well as the potential formation of a hydrogen bond with R13 (Fig. 7). The importance of a Thr residue at the (-) face of an α -CTx-interacting nAChR subunit has precedents. In the $\beta 2$ subunit, T59, which is two residues away from the homologous position to $\alpha 9T61$ (Fig. 1B), is a determinant of selectivity for α -CTx MII on the $\alpha 3\beta 2$ nAChR (Harvey et al., 1997), and a critical residue in off-rate kinetics of α -CTx BuIA on the $\beta 2$ subunit (Shiembob et al., 2006).

Taken together, our results strongly support the existence of an additional binding site for ACh in the $\alpha 9\alpha 10$ nAChR between the $\alpha 10(+)/\alpha 9(-)$ interface (Fig. 9). This $\alpha 10(+)/\alpha 9(-)$ interface is the high affinity binding site for α -CTx RgIA binding and consistent with a recent study, which also suggested a similar interaction for α -CTx Vc1.1 with the $\alpha 10(+)/\alpha 9(-)$ interface of the rat $\alpha 9\alpha 10$ nAChR (Yu et al., 2013). Conotoxins that bind the $\alpha 9\alpha 10$ nAChR are being considered as potential pain therapeutics (Bufalo et al., 2014; Di Cesare Mannelli et al., 2014; McIntosh et al., 2009) and understanding the subunit determinants of α -CTx interaction with the $\alpha 9\alpha 10$ nAChR may facilitate further development of such compounds.

Author Contribution:

Participated in research design: Azam and McIntosh

Conducted experiments: Azam and Papakyriakou

Contributed new reagents or analytic tools: Azam, Papakyriakou, Zouridakis, Giastas, Tzartos and McIntosh

Performed data analysis: Azam, Papakyriakou, Giastas, Zouridakis and Tzartos

Wrote or contributed to the writing of the manuscript: Azam, Papakyriakou, Zouridakis, Giastas, Tzartos and McIntosh

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Footnotes:

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Figure legends

Figure 1. Sequence alignments of rat $\alpha 9$ and $\alpha 10$ AChR subunits (A) Amino acid sequence alignment of the ECDs of rat $\alpha 9$ and $\alpha 10$ subunits. The $\alpha 9$ and $\alpha 10$ residues studied are indicated by arrows. Alignment was performed with MacVector 10.5.1 ClustalW alignment. (B) Sequence alignments of the α -CTx RgIA-interacting rat $\alpha 9$ and $\alpha 10$ domains with other nAChR α and β subunits. The residues in the $\alpha 10(+)$ and $\alpha 9(-)$ faces found to interact with α -CTx RgIA in this study are highlighted. Letters r and h stand for rat and human, respectively.

Figure 2. Dose response curves for α -CTx RgIA block of wild-type and mutant $\alpha 9\alpha 10$ nAChRs. (A) Mutation of (+) face residues in the $\alpha 9$ subunit did not affect α -CTx potency, whereas mutations in the (-) face residues decreased the potency of α -CTx RgIA. (B) Mutations of the (+) residues of the $\alpha 10$ subunit decreased α -CTx RgIA potency, whereas mutations of the (-) face residues did not. Values are mean \pm SEM from at least three different oocytes.

Figure 3. Dose response curves for α -CTx RgIA on rat $\alpha 9\alpha 10$, rat $\alpha 7$ and rat $\alpha 7$ mutants $\alpha 7S59T$ and $\alpha 7L119D$. There was an approximately 10-fold gain in potency of RgIA on the $\alpha 7L119D$ receptor mutant. The concentrations of ACh used were 100 μ M for $\alpha 9\alpha 10$ and 200 μ M for $\alpha 7$ and $\alpha 7$ mutants. Data are mean \pm SEM from at least three separate oocytes.

Figure 4: Agonist dose-response curves for $\alpha 9$ and $\alpha 10$ mutant receptors. Responses to a brief pulse of ACh on $\alpha 9\alpha 10$ nAChR mutants were measured as described in *Materials and Methods*. Data are mean \pm SEM from at least three separate oocytes.

Figure 5. (A) Ribbon representation of the X-ray crystal structure of the monomeric human $\alpha 9$ ECD (PDB ID: 4UXU) employed as template for the homology modeling of the rat $\alpha 9$ and $\alpha 10$ subunits. (B) Representative structure from the solution NMR conformational ensemble of α -CTx RgIA (PDB ID: 2JUQ). The side chains are shown in stick representation with C in green, N in blue, O in red and S in yellow. (C) Molecular model of the ECD of the rat $(\alpha 9)_2(\alpha 10)_3$ complex with α -CTx RgIA (green spheres). The arrangement of subunits is based on the X-ray crystal structure of *Aplysia californica* AChBP in complex with α -CTx ImI (PDB ID: 2BYP). α -CTx RgIA was superimposed with α -CTx ImI at the two $\alpha 10/\alpha 9$ ligand binding sites. (D) Side-view of the $\alpha 10/\alpha 9$ binding site with bound α -CTx RgIA (stick representation), where $\alpha 10(+)$ is designated as the principal subunit and $\alpha 9(-)$ as the complementary subunit.

Figure 6. (A) Representative structure of the top-populated conformational cluster from the MD simulations of the rat $(\alpha 9)_2(\alpha 10)_3$ complex with α -CTx RgIA indicating the position of the three $\alpha 10(+)$ residues E197, P200 and D201. (B) Close-up view of the $\alpha 10/\alpha 9$ binding interface illustrating the interactions between R7 and R11 of α -CTx RgIA, and the $\alpha 10(+)$ residues E197, P200 and D201. Residues from the $\alpha 10$ subunit are shown with carbon atoms in cyan and all other colors are as in Fig. 5. (C) Plot of the distance between R11-C^ζ and E197-C^δ during the course of the MD production runs. (D)

Plot of the distance between the R7- N^{η1} atom and P200-O (black line) or Q200-O (red line) in the α9α10P200Q mutant.

Figure 7. (A) Close-up view of the α-CTx binding interface from a representative structure of the rat (α9)₂(α10)₃ ECD complex with α-CTx RgIA, illustrating the interactions between R9 of α-CTx RgIA and D121, which forms a stable salt bridge interaction with R59 at the α9(-) subunit. Residues from α9 subunit are shown with carbon atoms in orange and all other colors are as in Fig. 5. (B) Close-up view of the second α10/α9 binding interface from a snapshot of the MDs of the rat (α9)₂(α10)₃ complex with α-CTx RgIA, illustrating the potential hydrogen bonding interactions of R13 of α-CTx RgIA with R59 and T61 at the α9(-) subunit. (C) Plot of the distance between R9-C^ζ and D121-O^δ from the first α10/α9 site as function of simulation time. (D) Plot of the distance between the C-terminus R13-C and R59-C^ζ from the second α10/α9 site as a function of simulation time. (E) For the same site, plot of the distance between R13-N^ε and T61-O^γ during the course of the MDs.

Figure 8. Close-up view of the α10/α9 binding interface from a representative structure of the MD simulations of rat (α9)₂(α10)₃ complex with ACh. The interacting residues within 4.5 Å from the ligand (green carbon atoms) are shown with cyan and orange carbon atoms for α10(+) and α9(-) subunits, respectively.

Data Supplement 1: Representative model of the ECD of rat (α9)₂(α10)₃ nAChR complex with α-CTx RgIA, selected from the highest populated cluster of 5,000

snapshots, derived from 100-ns unrestrained MD simulations. Chains A, B and D are the $\alpha 10$ subunits, chains C and E are the $\alpha 9$ subunits and RgIA is bound at the two $\alpha 10(+)/\alpha 9(-)$ binding sites (chains F and G). Water molecules and counter ions have been removed from the simulation system.

Data Supplement 2: Representative model of the ECD of rat $(\alpha 9)_2(\alpha 10)_3$ nAChR complex with ACh, selected from the highest populated cluster of 5,000 snapshots, derived from 100-ns unrestrained MD simulations. Chains A, B and D are the $\alpha 10$ subunits, chains C and E are the two $\alpha 9$ subunits, and the five ACh bound molecules are in chain L. Water molecules and counter ions have been removed from the simulation system.

Table 1: IC₅₀s and Hill coefficients for α -CTx RgIA on rat α 9 mutant receptors. The mutations making the greatest difference in toxin potency are in bold. CI: confidence interval. ND: not determined.

nAChR mutation	IC₅₀ (nM) RgIA (95% CI)	Hill coefficient
α 9 α 10	1.49 (1.13-1.95)	1.24 \pm 0.19
α9T61Iα10	2,610 (1,230-5,330)	0.71 \pm 0.16
α9T61Eα10	28.6 (17.1-47.6)	0.51 \pm 0.06
α9D121Lα10	> 1,000	ND
α 9E197Q α 10	1.15 (0.77-1.73)	0.61 \pm 0.07
α 9P200Q α 10	2.72 (2.13-3.46)	0.99 \pm 0.09
α 9D201N α 10	2.61 (2.08-3.26)	0.89 \pm 0.07

Table 2: IC₅₀s and Hill coefficients for α -CTx RgIA on rat α 10 mutant receptors. The mutations making the greatest difference in toxin potency are in bold. CI: confidence interval.

nAChR mutation	IC₅₀ (nM) RgIA (95% CI)	Hill coefficient
α 9 α 10	1.49 (1.13-1.95)	1.24 \pm 0.19
α 9 α 10E61I	2.0 (1.24-3.21)	0.78 \pm 0.12
α 9 α 10E61T	3.54 (2.81-4.47)	1.17 \pm 0.13
α 9 α 10D121L	1.36 (1.19-1.61)	1.14 \pm 0.09
α9α10E197Q	37.5 (26.6-52.8)	1.17 \pm 0.15
α9α10P200Q	466 (176-1,230)	0.55 \pm 0.13

Table 3: EC₅₀s for ACh dose response curves on α 9 and α 10 mutants that affected α -CTx RgIA potency. Values are mean \pm SEM from at least three different oocytes. CI: confidence interval.

nAChR	ACh EC₅₀ (95% CI) μM	Hill slope
α 9 α 10	10.1 (8.68-11.8)	1.2 \pm 0.1
α 9T61I α 10	10.7 (8.90-12.9)	1.4 \pm 0.2
α 9D121L α 10	323 (238-437)	1.8 \pm 0.4
α 9 α 10E197Q	193 (145-257)	0.54 \pm 0.04
α 9 α 10P200Q	419 (369-477)	0.81 \pm 0.04

Figure 1

A

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ra0 1  R N K Y R Q K L F S D L L E D W S S A L R P V D T D A V L N V T L Q V T L S Q I L D M D E R N Q 50
ra10 1 A E G R L A H K L F R L L A N V T I S A L R P V A D T D Q T L N V T L Q V T L S Q I L D M D E R N Q 50
      A G . A K L F D L F Y . S A L R P V D T D L N V T L V T L S Q I D M D E R N Q

ra9 31  I L A Y L W I R Q Y N H D A Y I I W R D Q T D R L D S I R I P S D I L V W R P D I V L Y N K A D D 100
ra10 31 V L L I L W I R Q E I I D A Y L H W D P K A H G D L L A L R I P S R L V W R P D I V L Y N K A D I T 100
      L I Y I W I R Q E D A Y L W D I L D I R I P S L V W R P D I V L Y N K A D

ra9 101 E S S E P V N I N V V I R V D G L I T W S P A I T K S S C V W D T Y P F F D S Q Q N I L F G S 150
ra10 101 Q P P A S A S I N V V V R H D G A V R W D A P A I T R S S C R V D V S A L P F P D A Q R I G L I F G S 150
      . T R V V . R D G . . W D P A I T . S S C V D V . F P F D Q C L I F G S

ra9 151 W T Y W G N Q V D I F N A L D S G D I S I D F I E D V E W E V H G M P A V K N M I S Y G C C S E P Y P 200
ra10 151 W T H G G H I I D V R P R C T S A S I A D D V H W E N V N I G W P A R R R I T I G G C S E P Y P 200
      W T G Q . D . . S . L D F . E V E W V G M P A V . Y G C C S E P Y P

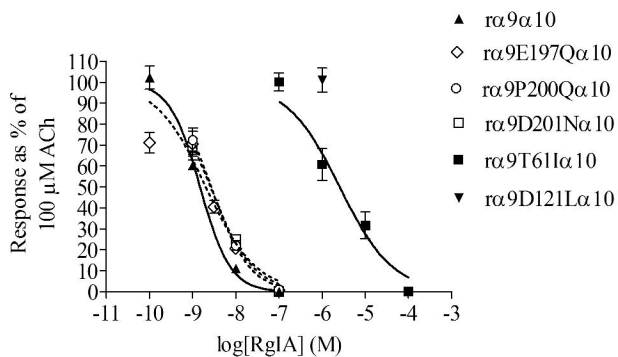
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ra10 201 D V T F T I L L R R R 210
      D V T F T I L L R R R
  
```

B

	190	200		60		
ra10	LTYGCCS	SPYR	LVTFFT	ra9	YLWIRQ	WHDAY
ra9	ISYGCCS	SPYR	LVTFFT	ra10	YLWIRQ	WTDAY
ha9	ISYGCCS	SPYR	LVTFFT	ha9	YLWIRQ	WHDAY
ha10	LTYGCCS	SPYR	LVTFFT	ha10	YLWIRQ	WTDAY
ha1	VTYSCCP	TP	LLITYH	ha7	NIWLQM	WTDHY
ha2	KRYDCCA	IYR	LVTYA	hb2	NVWLQ	WEDYR
ha3	IKYNCCS	IYR	LVITYS	hb4	NVWLKQ	WTDYR
ha4	RKYECCA	IYR	LVITYA			
ha5	RTDSCC	WYR	LVITYS		120	
ha6	IKYNCCS	IYR	LVITYS	ra9	DGLITW	SPAIT
ha7	RFYECCK	SPYR	LVTFFT	ra10	DGAVRW	APAIT
				ha9	DGLITW	APAIT
				ha10	DGAVRW	APAIT
				ha7	SGHCQY	PPGIF
				hb2	DGSLPW	PPAIY
				hb4	NGSVLW	PPAIY

Figure 2

A



B

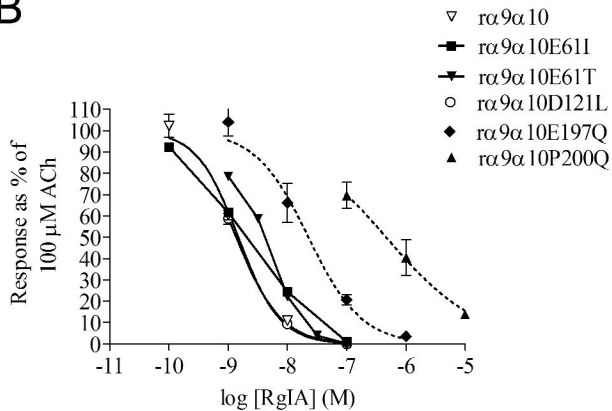


Figure 3

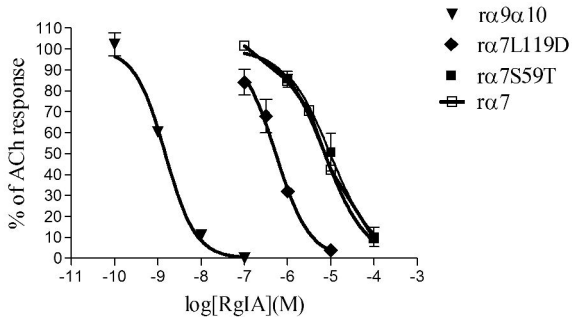


Figure 4

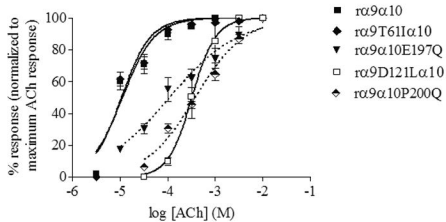


Figure 5

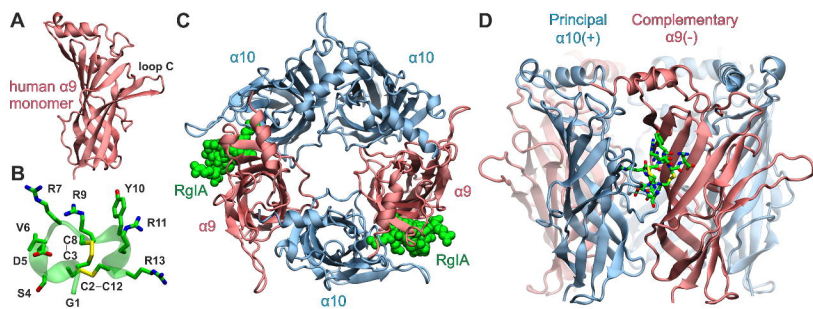


Figure 6

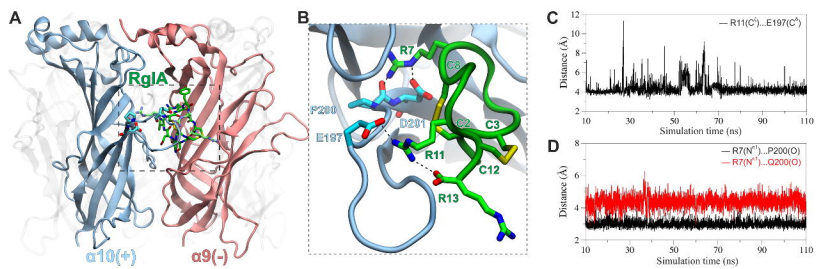


Figure 7

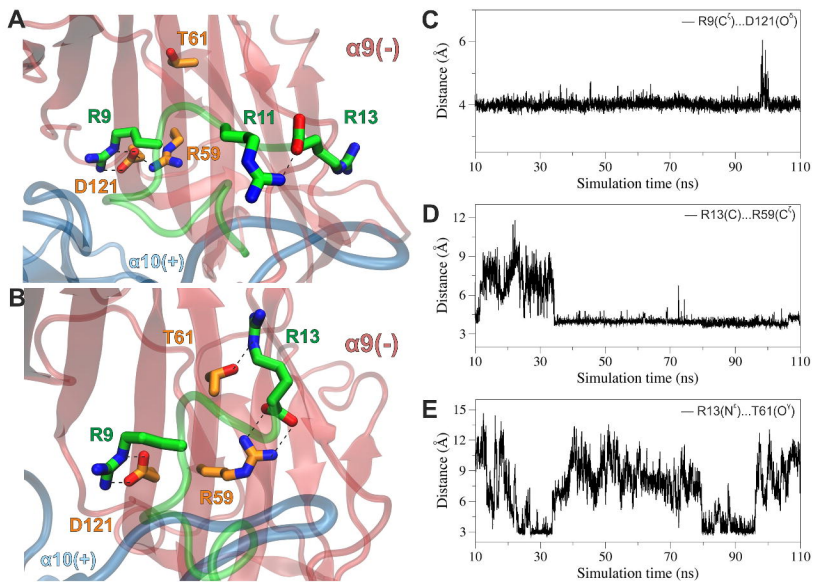


Figure 8

