

# Corrections to “Molecular interaction of $\alpha$ -conotoxin RgIA with the rat $\alpha 9\alpha 10$ nicotinic acetylcholine receptor”

In the above article [Azam L, Papakyriakou A, Zouridakis M, Giastas P, Tzartos SJ, McIntosh JM, (2015) *Mol Pharmacol* **87**:855–864]. Since publication of the manuscript it has come to our attention that the the PDB structure of a point mutant of RgIA (RgIA(P6V)) was inadvertently used instead of RgIA for molecular modeling simulations. The modeling calculations have been redone with RgIA and the corresponding figures and text have been corrected accordingly. Modeling differences based on the native peptide compared to the point mutation did not affect our original conclusions that were obtained from pharmacological experiments.

Revised sections of the Methods, Results and Discussion are included. The corrected Figures can be found within the original article. The authors regret this error and any inconvenience it may have caused.

**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  $\alpha$ -CTx RgIA (PDB ID 2JUT). The side chain atoms 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  $\alpha$ -CTx RgIA (green spheres). The arrangement of subunits is based on the X-ray crystal structure of *Aplysia californica* AChBP in complex with  $\alpha$ -CTx ImI (PDB ID 2BYP).  $\alpha$ -CTx RgIA was superimposed with  $\alpha$ -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  $\alpha$ -CTx RgIA (stick representation), where  $\alpha 10(+)$  is designated as the principal subunit and  $\alpha 9(-)$  as the complementary subunit.

**Figure 6.** (A) One of the two  $\alpha 10(+)/\alpha 9(-)$  binding interfaces of the rat  $(\alpha 9)_2(\alpha 10)_3$  in complex with  $\alpha$ -CTx RgIA, taken from the centroid of the top-populated conformational cluster, that was extracted from 100-ns MD simulations. (B) Close-up view of the  $\alpha 10/\alpha 9$ -binding interface illustrating the hydrogen bonding interactions between R7 and R11 of  $\alpha$ -CTx RgIA and the  $\alpha 10(+)$  residues E197, P200, and D201 with dashed lines, and the alkyl-aryl interactions between P6 and W151 with dotted lines. Residues from the  $\alpha 10$  subunit are shown with carbon atoms in cyan, and all other colors as in Fig. 5. (C) Plot of the distance between R11-C $^{\zeta}$  and E197-C $^{\delta}$  as a function of simulation time within the two ligand-binding sites of the  $(\alpha 9)_2(\alpha 10)_3$  model. (D) Plot of the distance between the R7-N $^{\eta 1}$  and the carbonyl group at position 200 as a function of time in the wild-type receptor (black line) and the P200Q mutant (red line).

**Figure 7.** (A) Close-up view of the  $\alpha 9(-)$  face from the representative model of the rat  $(\alpha 9)_2(\alpha 10)_3$  ECD complex with  $\alpha$ -CTx RgIA, illustrating the hydrogen bonding interactions between R9, Y10 and R13 of  $\alpha$ -CTx RgIA (green C atoms) and residues from the  $\alpha 9$  subunit shown with orange C atoms, while all other colors are as in Fig. 5. (B) Plots of the distance between R9-C $^{\zeta}$  and D121-C $^{\delta}$  during the course of the MD simulations of the native  $(\alpha 9)_2(\alpha 10)_3$  complex. (C) Plots of the distance between Y10-OH and T119-OH, and (D) between D121-C $^{\delta}$  and R59-C $^{\zeta}$  as a function of simulation time in the native model (black lines) and the  $\alpha 9$ T61I mutant (red lines).

**Figure S2.** (A, B) Positional root-mean-square deviation (RMSD) from the initial model of rat  $(\alpha 9)_2(\alpha 10)_3$  ECD complex with  $\alpha$ -CTx RgIA during the equilibration (A) and the production (B) phase, calculated for the backbone atoms of the receptor (black line) and the two bound  $\alpha$ -CTxs (red and blue lines). (C, D) Radius of gyration (Rg) of the receptor during the course of the equilibration (C) and the production (D) phase of the MD simulations.

**Figure S4.** Plots of selected intermolecular and intramolecular interactions as a function of simulation time at the  $\alpha 10(+)$  face (left panels) and  $\alpha 9(-)$  face (right panels) extracted from the MD simulations of the rat  $(\alpha 9)_2(\alpha 10)_3$  ECD complex with  $\alpha$ -CTx RgIA.

## Methods

**Molecular modeling methods.** The molecular model of the extracellular domain (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 (**Supplemental 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  $\alpha$ -CTx RgIA was taken from the representative conformation (model 1) of its NMR structure (PDB ID: 2JUT) (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  $\alpha$ -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 was superimposed with chains C and E, so that two  $\alpha 10(+)/\alpha 9(-)$  binding interfaces were generated between chains A(+)/E(-) and D(+)/C(-). The final model of the rat  $(\alpha 9)_2(\alpha 10)_3$  complex with  $\alpha$ -CTx RgIA was prepared by superimposing the NMR structure of RgIA with chains F and I of  $\alpha$ -CTx ImI in the AChBP complex. The  $\alpha 9\alpha 10$  P200Q and T61I mutants were prepared by changing P200 of a chain A( $\alpha 10$ ) to Q200, and T61 of chain C( $\alpha 9$ ) to I61 in the final model. The model of the rat  $(\alpha 9)_2(\alpha 10)_3$  complex with ACh was prepared by a similar procedure using 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.

## Results

**Molecular Modeling Studies of the Complex of  $\alpha 9\alpha 10$  ECD with  $\alpha$ -CTx RgIA.** To gain insight into the molecular basis of the interaction between the rat nAChR and  $\alpha$ -CTx RgIA, we employed MD calculations of the ECD of the rat  $(\alpha 9)_2(\alpha 10)_3$  nAChR complex with  $\alpha$ -CTx RgIA. Our model was based on the recent X-ray crystal structure of the monomeric state of the ECD of the human  $\alpha 9$  nAChR (Fig. 5A) (Zouridakis et al., 2014) and the solution NMR structure of  $\alpha$ -CTx RgIA (Fig. 5B) (Ellison et al., 2008). Molecular models of the highly homologous rat  $\alpha 9$  and  $\alpha 10$  subunits were assembled in the pentameric state (Fig. 5, C and D) on the basis of the AChBP complex with  $\alpha$ -CTx ImI, as described in *Materials and Methods*. Two  $\alpha$ -CTx molecules were modeled in the two  $\alpha 10(+)/\alpha 9(-)$  binding sites (Fig. 5C), and unrestrained MD simulations in explicit solvent were carried out (Supplemental Material 1). The stability of the systems during the course of the MD simulations is demonstrated by the equilibrated values of the root-mean-square deviations from the initial conformation and the radius of gyration of the receptor (Supplemental Figs. 2 and 3). Although the initial orientation of the two bound  $\alpha$ -CTx ligands was similar, our MD simulations displayed intermolecular and intramolecular interactions that varied as a function of time. In general, the long side chains of R7 and R9 in  $\alpha$ -CTx RgIA exhibited remarkable stability in their interactions with the  $\alpha 10(+)$  and  $\alpha 9(-)$  face, respectively, followed by R11 and the C-terminal R13 that displayed the highest mobility. The two mutations,  $\alpha 10$ P200Q and  $\alpha 9$ T61I, introduced at the models of rat  $(\alpha 9)_2(\alpha 10)_3$  complex with  $\alpha$ -CTx RgIA revealed the potential perturbation of the  $\alpha 10(+)/\alpha 9(-)$  ligand binding site, as discussed below.

At the  $\alpha 10(+)$  side (Fig. 6A), E197 displayed a salt bridge interaction with R11 of  $\alpha$ -CTx RgIA (Fig. 6B). This interaction was stable throughout the total 100-ns production MD run in only one of the two binding sites of our model, (Fig. 6C), an observation indicating the high mobility of the two solvent exposed partners. However, impairment of the  $\alpha 10$ E197 electrostatic interaction with R11 of  $\alpha$ -CTx could explain the significant decrease in the sensitivity of the  $\alpha 9\alpha 10$ E197Q mutant to  $\alpha$ -CTx RgIA (Table 2). R7 of  $\alpha$ -CTx RgIA exhibited electrostatic interactions with D201, an interaction that was stabilized by an intramolecular salt bridge with D5 (Fig. 6B). Although we were not able to obtain functional expression

of the  $\alpha 10D201N$  mutation, our data indicated that the  $\alpha 10D201$  interaction with R7 was stable at the timescale of the MD simulations (Supplemental Fig. S4), suggesting the importance of the negatively charged Asp residue at this position for the binding of  $\alpha$ -CTx RgIA. The arrangement of residues  $\alpha 10D201$  and D5 was further stabilized through a salt bridge with R188 and a hydrogen bond with the phenolic group of Y192, respectively (Supplemental Fig. S4). In addition, the side chain of R7 displayed a stable hydrogen bond with the carbonyl group of P200 (Fig. 6, B and D). Since the predicted interaction of R7 with the main chain at position 200 of  $\alpha 10$  cannot readily explain the observed 300-fold reduction in the potency of  $\alpha$ -CTx RgIA for the  $\alpha 9\alpha 10P200Q$  mutant, we carried out simulations of the same model, but with a single P200Q mutation in one of the two  $\alpha 10(+)/\alpha 9(-)$  sites. 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 rationalized by the higher flexibility of Q200 compared to P200, which allows a rotation of the backbone carbonyl group away from R7. Therefore, the significant decrease in the sensitivity of the  $\alpha 9\alpha 10P200Q$  mutant to  $\alpha$ -CTx RgIA can be attributed to destabilization of the R7–P200 interaction at the  $\alpha 10(+)$  ligand-binding site. Another interesting observation is the  $CH_2 \cdots \pi$  interaction of P6 of  $\alpha$ -CTx RgIA with the indole moiety of  $\alpha 10W151$  (Supplemental Fig. S4).

At the  $\alpha 9(-)$  face, D121 formed a stable salt bridge interaction with R9 of  $\alpha$ -CTx RgIA throughout the MD simulations in both binding sites (Fig. 7, A and B), an interaction that could be responsible for the >670-fold decrease in the potency of  $\alpha$ -CTx RgIA in the  $\alpha 9D121I\alpha 10$  mutant (Table 1). In one  $\alpha 10(+)/\alpha 9(-)$  site, Y10 of  $\alpha$ -CTx RgIA displayed a stable hydrogen-bond interaction with T119, which was not observed in the simulations of the  $(\alpha 9)_2(\alpha 10)_3$  model with the  $\alpha 9T61I\alpha 10$  mutation (Fig. 7, A and C). The stable interaction between Y10 and T119 was accompanied by a hydrogen bond between the side chain hydroxyl groups of T119 and T61 (Supplemental Fig. S4). At the same site, D121 formed a stable hydrogen bond with R59, an intramolecular interaction that was also apparent at the X-ray crystal structure of the monomeric human  $\alpha 9$  ECD (Zouridakis et al., 2014). In addition, R59 was hydrogen bonded with the side chain of Q36 (Fig. 7A and Supplemental Fig. S4). The topology of these interlinked interactions was stable only throughout the course of the MD simulations of the native receptor, in contrast to the simulations of the  $\alpha 9T61I\alpha 10$  mutant (for example see Fig. 7D). Although we did not observe a direct interaction between T61 and any residue of  $\alpha$ -CTx RgIA, our data imply that the  $\alpha 9T61I\alpha 10$  mutation affects the sensitivity of the receptor due to disruption of these interactions. Another possibility for the experimental effect of the  $\alpha 9T61I\alpha 10$  mutation (Fig. 2; Table 3) could be the interruption of a hydrogen bond between R13 of  $\alpha$ -CTx RgIA and the side chain of  $\alpha 9T34$ , an interaction that was only observed in the simulations of the native receptor (Fig. 7A). This hydrogen bond was mediated through a salt bridge interaction of the C-terminal group of R13 and the side chain of  $\alpha 9R113$  (Supplemental Fig. S4). However, both of these interactions were short-lived as a result of the high mobility demonstrated by R13 of  $\alpha$ -CTx RgIA in all our MD simulations, and thus this interaction should be considered tentative.

## Discussion

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.  $\alpha 9T61$  was previously shown (Azam and McIntosh, 2012) to confer ~300-fold higher potency of  $\alpha$ -CTx RgIA on the rat versus human  $\alpha 9$  subunit, which instead has an Ile residue at this position (Fig. 1B). In addition, when we mutated  $\alpha 9T61$  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  $\alpha$ -CTx RgIA was observed (Table 1), confirming that  $\alpha 9$  confers the  $(-)$  face of  $\alpha$ -CTx binding site in the  $\alpha 9\alpha 10$  nAChR. Our MD simulations of the rat  $\alpha 10(+)/\alpha 9(-)$ -binding interface suggest that the  $\alpha 9T61I\alpha 10$  mutation might probably disrupts a hydrogen bonding network comprising R9 and Y10 of  $\alpha$ -CTx RgIA, and D121–R59–Q36 and T119–T61 of the  $\alpha 9(-)$  face (Fig. 7A). The importance of a Thr residue at the  $(-)$  face of an  $\alpha$ -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  $\alpha$ -CTx MII on the  $\alpha 3\beta 2$  nAChR (Harvey et al., 1997), and a critical residue in off-rate kinetics of  $\alpha$ -CTx BuIA on the  $\beta 2$  subunit (Shiembob et al., 2006).