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## Abstract

$\alpha 9\alpha 10$  nicotinic acetylcholine receptors (nAChRs) putatively exist at different stoichiometries. We systematically investigated the molecular determinants of  $\alpha$ -conotoxins Vc1.1, RgIA#, and PeIA inhibition at hypothetical stoichiometries of the human  $\alpha 9\alpha 10$  nAChR. Our results suggest that only Vc1.1 exhibits stoichiometric-dependent inhibition at the  $\alpha 9\alpha 10$  nAChR. The hydrogen bond between N154 of  $\alpha 9$  and D11 of Vc1.1 at the  $\alpha 9(+)$ - $\alpha 9(-)$  interface is responsible for the stoichiometric dependent potency of Vc1.1.

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# **Molecular determinants conferring the stoichiometric-dependent activity of $\alpha$ -conotoxins at the human $\alpha 9\alpha 10$ nAChR subtype**

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## ABSTRACT

$\alpha 9\alpha 10$  Nicotinic acetylcholine receptors (nAChRs) putatively exist at different stoichiometries. We systematically investigated the molecular determinants of  $\alpha$ -conotoxins Vc1.1, RgIA#, and PeIA inhibition at hypothetical stoichiometries of the human  $\alpha 9\alpha 10$  nAChR. Our results suggest that only Vc1.1 exhibits strong stoichiometric-dependent inhibition at the  $\alpha 9\alpha 10$  nAChR. The hydrogen bond between N154 of  $\alpha 9$  and D11 of Vc1.1 at the  $\alpha 9(+)$ - $\alpha 9(-)$  interface is responsible for the stoichiometric-dependent potency of Vc1.1.

## Introduction

Nicotinic acetylcholine receptors (nAChRs) are cation-selective pentameric ligand-gated ion channels belonging to the family of Cys-loop receptors, which also includes  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>), glycine, and serotonin (5-HT<sub>3</sub>) receptors.<sup>1</sup> The vertebrate nAChRs are homo/heteromeric assemblies of  $\alpha$ 1- $\alpha$ 10,  $\beta$ 1- $\beta$ 4,  $\gamma$ ,  $\delta$  or  $\epsilon$  subunits. Although the nAChRs are generally featured in the nervous system, they are also expressed in non-neuronal cells participating in various physiological events and they are important targets for drug design.<sup>2</sup>

The first crystal structure of any heteromeric nAChRs, the human(h)  $\alpha$ 4 $\beta$ 2 nAChR subtype, reveals the overall architecture consisting of a large extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD) (Figure 1A).<sup>3</sup> The ligand binding site is located at the ECD interface of two adjacent subunits, comprising of (+) (principal) and (−) (complementary) components. For h $\alpha$ 4 $\beta$ 2 nAChR, the  $\alpha$ 4 subunit loops A, B and C form the (+) site, and the  $\beta$ 2 subunit  $\beta$ -sheet contributes to the (−) site (Figure 1B). Five subunits of the nAChR circle a conducting pore (Figure 1C) where a gate consisting of hydrophobic residues, such as Leu and Ile, is located in the middle of the TMD.

Heteromeric  $\alpha$ 9 $\alpha$ 10 nAChRs play an important physiological role in mediating olivocochlear and vestibular neurotransmission.<sup>4,5</sup> Transcripts/protein expression of  $\alpha$ 9 and/or  $\alpha$ 10 subunits have been reported in dorsal root ganglion neurons,<sup>6,7</sup> adrenal medulla,<sup>8</sup> and in other non-neuronal cells, such as skin keratinocytes, pituitary pars tuberalis, lymphocytes, macrophages and bladder urothelium.<sup>9–15</sup>

$\alpha$ 9-Containing nAChRs are potential targets for the therapy of several disorders or diseases such as ear disorders, chronic pain and pemphigus vulgaris.<sup>7,9,11,16,17</sup> In addition,  $\alpha$ 9-containing nAChRs are responsible for nicotine-induced transformation of normal human breast epithelial cells, and inducible overexpression of  $\alpha$ 9-nAChR substantially increased tumor growth.<sup>18</sup>

Conotoxins derived from the venom of *Conus* sea snails are pharmacologically valuable peptides with selective potency at nAChR subtypes.  $\alpha$ -Conotoxin antagonists of  $\alpha$ 9 $\alpha$ 10 nAChR such

as *C. victoriae* Vc1.1, *C. regius* RgIA (Figure 2), and *C. generalis* GeXIVA are short and well-folded peptides with analgesic effect in rat models of neuropathic pain.<sup>19,20</sup> In addition, PeIA from *C. pergrandis*, displays high selectivity for the  $\alpha 9\alpha 10$  nAChR subtype.<sup>21</sup> Similar to Vc1.1 and RgIA, PeIA is also a potent inhibitor of N-type calcium channels via GABA<sub>B</sub> receptor activation.<sup>22</sup>

Despite their specificity at inhibiting  $\alpha 9\alpha 10$  nAChRs, the binding site and the stoichiometry-dependent sensitivity of each  $\alpha$ -conotoxin are varied. The stoichiometry of rat(r)  $\alpha 9\alpha 10$  nAChR heterologously expressed in *Xenopus laevis* oocytes was initially suggested to consist of two  $\alpha 9$  and three  $\alpha 10$  subunits ( $(\alpha 9)_2(\alpha 10)_3$ ).<sup>23</sup> Based on this stoichiometry, Vc1.1 was postulated to bind preferentially at the  $\alpha 10(+)$ - $\alpha 9(-)$  interface.<sup>24</sup> However, Indurthi *et al.* proposed at least two stoichiometries (see Figure 1D,E), possibly  $(\alpha 9)_2(\alpha 10)_3$  and  $(\alpha 9)_3(\alpha 10)_2$ , with a high Vc1.1-sensitive  $\alpha 9(+)$ - $\alpha 9(-)$  binding site present in the  $(\alpha 9)_3(\alpha 10)_2$  composition, and two low Vc1.1-sensitive binding sites contributed by the  $\alpha 10(+)$ - $\alpha 9(-)$  and/or  $\alpha 9(+)$ - $\alpha 10(-)$  interfaces that are common in both subunit arrangements.<sup>25</sup> In contrast, RgIA exhibited no preference for the Vc1.1-high sensitivity binding site of the  $\alpha 9\alpha 10$  nAChR, and it was reported that RgIA has a high affinity for the  $\alpha 10(+)$ - $\alpha 9(-)$  interface.<sup>26</sup>

The molecular determinants that govern the binding site selectivity and stoichiometry-dependent sensitivity of Vc1.1, RgIA, and PeIA at  $\alpha 9\alpha 10$  nAChR, however, remain elusive and the activity of PeIA at different  $\alpha 9\alpha 10$  nAChR stoichiometries is yet to be reported. In this study, we performed comprehensive computational modeling based on the proposed high and low Vc1.1 sensitivity binding sites in  $(\alpha 9)_2(\alpha 10)_3$  and  $(\alpha 9)_3(\alpha 10)_2$  stoichiometries, in combination with mutagenesis study on h $\alpha 9\alpha 10$  nAChRs expressed in oocytes injected with varied  $\alpha 9:\alpha 10$  mRNA ratios. The sequences of Vc1.1, RgIA, and PeIA are remarkably different from each other despite their relatively potent antagonism of the  $\alpha 9\alpha 10$  nAChR. We aim to identify residues of the receptor and/or peptides responsible for the  $\alpha 9\alpha 10$  nAChR binding site selectivity and sensitivity of these peptides. This information is essential for the design of  $\alpha$ -conotoxin analogues with improved selectivity for a specific  $\alpha 9\alpha 10$  nAChR stoichiometry.

## Results and discussion

### *Molecular dynamics simulations*

Vc1.1 was postulated to inhibit  $\alpha 9\alpha 10$  nAChR by binding at a high affinity  $\alpha 9(+)-\alpha 9(-)$  interface and/or a low affinity  $\alpha 10(+)-\alpha 9(-)$  interface.<sup>24,25</sup> Both Vc1.1 binding sites share a common  $\alpha 9$  subunit  $(-)$  component, therefore, the determinants of Vc1.1 binding should be contributed by non-conserved residues of the  $\alpha 9$  and  $\alpha 10$  subunit  $(+)$  components. Overall, our molecular dynamics (MD) simulation model suggests, the binding modes of Vc1.1 at both  $\alpha 9(+)-\alpha 9(-)$  and  $\alpha 10(+)-\alpha 9(-)$  interfaces are similar with four salt-bridges formed involving charged residues of Vc1.1 (R7, D11, H12 and E14) and conserved residues of the h $\alpha 9$  and h $\alpha 10$  subunits (R81, R113, E197 and D201). The R7-D201, H12-E197, D11-R81 and E14-R113 hydrogen bonds (Figure 3A,B) are consistent with the previously proposed interaction of Vc1.1 with the  $\alpha 10$  subunit  $(+)$  site residues.<sup>24</sup>

In addition to the interaction with R81 of the  $\alpha 9$  subunit  $(-)$  component, D11 of Vc1.1 forms additional hydrogen bond with a non-conserved N154 at the  $\alpha 9(+)-\alpha 9(-)$  interface (Figure 3B). In comparison, the corresponding  $\alpha 10$  subunit residue G154 (Figure 3A) is incapable of direct interaction with Vc1.1. Other non-conserved  $\alpha 9$  and  $\alpha 10$  subunit  $(+)$  site residues at position 188 and 190 are postulated to have no contribution to the binding affinity of Vc1.1. Our model postulates residues  $\alpha 9$  N188 and  $\alpha 10$  R188 are not feasibly located to interact with Vc1.1, whereas residues  $\alpha 9$  I190 and  $\alpha 10$  L190 have similar side chains.

In this study, we used the R13-deleted analogue of RgIA (RgIA#) to probe its sensitivity to different stoichiometries of  $\alpha 9\alpha 10$  nAChRs. RgIA# has been reported to have comparable activity to the wild-type RgIA.<sup>27,28</sup> RgIA# has an internal salt-bridge formed between residues D5 and R7, involved in binding at both  $\alpha 10(+)-\alpha 9(-)$  and to  $\alpha 9(+)-\alpha 9(-)$  interfaces. At both binding sites, residues R7 and R9 of RgIA# are buried in the binding pockets forming salt-bridges with D201 and D121 of the  $(+)$  and  $(-)$  components, respectively (Figure 3C,D). Similarly, the conserved aspartate residues D201 and D121 were suggested to interact with RgIA residues R7 and R9, respectively, at the  $\alpha 10(+)-\alpha 9(-)$  interface.<sup>28</sup> It was also proposed that R11 of RgIA interacts with E197 of the  $\alpha 10$

subunit. However, residue R11 of RgIA# is solvent exposed and flexible in our MD simulations, and only occasionally approaching E197 of the  $\alpha 10$  and  $\alpha 9$  (+) components forming unstable salt-bridges.

The DPR (Asp-Pro-Arg) motif is conserved in  $\alpha$ -conotoxins ImI, RgIA# (or RgIA) and Vc1.1, and essential to their binding affinities.<sup>26,29–31</sup> However, this sequence epitope is not present in PeIA despite its higher potency at inhibiting the  $\alpha 9\alpha 10$  nAChR than Vc1.1 and RgIA# (Figure 2, S1). In PeIA, the side chain of H5 forms an internal hydrogen bond with C8 (Figure S2), which might be essential to stabilize the secondary structure of the peptide. No remarkable differences were observed in the binding of PeIA at the  $\alpha 10(+)-\alpha 9(-)$  and  $\alpha 9(+)-\alpha 9(-)$  interfaces. Similar to Vc1.1, PeIA residues H12 and E14 interact with residues E197 of the (+) and R113 of the (–) components, respectively (Figure 3E,F).

In contrast to Vc1.1, both RgIA# and PeIA do not interact with N154 of the  $\alpha 9$  subunit (+) component and for PeIA, an additional hydrogen bond is formed between residue N11 (D11 in Vc1.1) and T152 of the (+) component at both binding sites. Therefore, our model suggests lack of binding site preference between  $\alpha 10(+)-\alpha 9(-)$  and  $\alpha 9(+)-\alpha 9(-)$  for RgIA# and PeIA.

### ***Energy calculations***

Quantitative evaluation on the contribution of the principal site residues to Vc1.1, RgIA# and PeIA binding affinity was assessed using the MMGB/SA (molecular mechanics generalized Born/surface area) method (Figure S3). Conserved residues on both  $\alpha 9$  and  $\alpha 10$  subunit (+) sites such as Y95, W151, T152, Y192, C194, C195, E197 and Y199 significantly contribute to the binding affinity of Vc1.1, RgIA# and PeIA. Indeed, in our model the aromatic residues of the binding site form compact van der Waals contacts with the  $\alpha$ -helix of the  $\alpha$ -conotoxins, and the C192-C193 disulfide bond stacks with the C2-C8 disulfide bond of the  $\alpha$ -conotoxins. However, for the four (+) site non-conserved residues at positions 154, 188, 190 and 191, only residue 154 (N in  $\alpha 9$  subunit and G in  $\alpha 10$  subunit) showed significant difference in the binding energy upon interaction with Vc1.1 (Figure S3 A). The side chain of  $\alpha 9$  N154 contributes about  $-4$  kcal/mol, whereas  $\alpha 10$  G154 only



resulted in  $-0.19$  kcal/mol Vc1.1-binding energy shift. Binding energy calculations for RgIA# (Figure S3B) and PeIA (Figure S3C) showed minor contribution of  $\alpha 9$  N154 and  $\alpha 10$  G154 in the interactions with both  $\alpha$ -conotoxins.

RgIA was suggested to have a high affinity binding site at the rat  $\alpha 10(+)$ - $\alpha 9(-)$  interface based on the stoichiometry model of  $(\alpha 9)_2(\alpha 10)_3$  nAChR.<sup>26</sup> Our modeling studies showed comparable RgIA# binding energy at the principal subunits of  $\alpha 9(+)$  and  $\alpha 10(+)$  hence suggesting RgIA# has no binding preference for either  $\alpha 9(+)$ - $\alpha 9(-)$  or  $\alpha 10(+)$ - $\alpha 9(-)$  interfaces of the  $(\alpha 9)_3(\alpha 10)_2$  nAChR.

Overall, our computational studies suggest Vc1.1 possesses binding preference for the  $\alpha 9(+)$ - $\alpha 9(-)$  interface than the  $\alpha 10(+)$ - $\alpha 9(-)$  interface. Binding of RgIA# and PeIA at the  $\alpha 9\alpha 10$  nAChR on the other hand, is less dependent on the stoichiometry. A hydrogen-bond is formed between  $\alpha 9$  N154 and Vc1.1 D11 at the  $\alpha 9(+)$ - $\alpha 9(-)$  interface and most importantly, this interaction is absent in RgIA# and PeIA binding models at the aforementioned  $\alpha 9\alpha 10$  nAChR site.

### ***Electrophysiology***

Injection of biased  $\alpha 9$  to  $\alpha 10$  subunit mRNA ratios resulted in putative  $(\alpha 9)_3(\alpha 10)_2$  and  $(\alpha 9)_2(\alpha 10)_3$  stoichiometries of the  $\alpha 9\alpha 10$  nAChRs heterologously expressed in *X. laevis* oocytes.<sup>25</sup> Consequently, a high-sensitivity Vc1.1 binding site was proposed at the  $\alpha 9(+)$ - $\alpha 9(-)$  interface present only in the  $(\alpha 9)_3(\alpha 10)_2$  stoichiometry, and a low-sensitivity Vc1.1 binding site contributed by the  $\alpha 10(+)$ - $\alpha 9(-)$  interface that exists in both stoichiometries. Most importantly, the stoichiometry of the  $\alpha 9\alpha 10$  nAChR expressed at 1:1 (or 10:1) and 1:3 ratios of  $\alpha 9$ : $\alpha 10$  mRNA was proposed to be  $(\alpha 9)_3(\alpha 10)_2/(\alpha 9)_4(\alpha 10)_1$  and  $(\alpha 9)_2(\alpha 10)_3$  nAChRs, respectively.

To validate our prediction that the hydrogen bond interaction between Vc1.1 D11- $\alpha 9$  and N154 is the determinant of Vc1.1 binding preference at the  $\alpha 9(+)$ - $\alpha 9(-)$  interface, residue N154 of the  $\alpha 9$  subunit was mutated to the corresponding  $\alpha 10$  subunit G154. We determined the biological activity of Vc1.1, RgIA#, PeIA and, analogues Vc1.1[D11N] and Vc1.1[PeIA] at heterologously co-expressed  $\alpha 9/\alpha 9$ [N154G] and  $\alpha 10$  subunits in *X. laevis* oocytes, injected at varying mRNA ratios.

Concentration-response relationships for Vc1.1, RgIA# and PeIA inhibition of  $\alpha 9\alpha 10$  nAChR, expressed from an  $\alpha 9:\alpha 10$  mRNA ratio of 1:1, gave half-maximal inhibitory concentration ( $IC_{50}$ ) values of  $1.0 \pm 0.1 \mu M$  ( $n = 10-16$ ),  $248.7 \pm 19.2 nM$  ( $n = 5-6$ ) and  $22.2 \pm 1.6 nM$  ( $n = 6-8$ ), respectively (Figure S1). Subsequent experiments were carried out using  $1 \mu M$  Vc1.1,  $300 nM$  RgIA# and  $30 nM$  PeIA, close to the  $IC_{50}$  values of the  $\alpha$ -conotoxins at the  $\alpha 9\alpha 10$  nAChR.

At  $\alpha 9:\alpha 10$  mRNA ratios of 3:1 putatively resulting in  $(\alpha 9)_3(\alpha 10)_2$  and/or  $(\alpha 9)_4(\alpha 10)_1$  nAChRs,  $1 \mu M$  Vc1.1 inhibited ACh-evoked currents by  $65.8 \pm 1.8\%$  ( $n = 16$ ), respectively (Figure 4A). However, at  $\alpha 9:\alpha 10$  mRNA ratio of 1:3, presumably forming the  $(\alpha 9)_2(\alpha 10)_3$  nAChR stoichiometry,  $1 \mu M$  Vc1.1 inhibited ACh-evoked currents by  $25.5 \pm 1.8\%$  ( $n = 15$ ). Similarly, Vc1.1 antagonized only ~20% of the ACh-evoked currents mediated by  $\alpha 9[N154G]\alpha 10$  nAChRs at 1:3 ( $19.5 \pm 1.0\%$ ,  $n = 12$ ) and 3:1 ( $21.8 \pm 2.2\%$ ,  $n = 14$ )  $\alpha 9:\alpha 10$  mRNA ratios (Figure 4A).

Vc1.1 concentration-response relationships for  $\alpha 9\alpha 10$  nAChRs expressed from biased  $\alpha 9:\alpha 10$  ratios revealed  $IC_{50}$  values of  $0.75 \mu M$  at 3:1 ratio compared to  $3.57 \mu M$  for 1:3 ratio (Figure S3, Table 1). N154G mutation to the  $\alpha 9$  subunit, however, reduced the potency of Vc1.1 with  $IC_{50}$ 's of  $11.25 \mu M$  and  $17.02 \mu M$  for  $\alpha 9[N154G]\alpha 10$  nAChRs at 1:3 and 3:1 ratios, respectively. Injections of higher  $\alpha 9$  to  $\alpha 10$  mRNA ratios resulted in greater Vc1.1 inhibition at the expressed  $\alpha 9\alpha 10$  nAChRs compared to injections with excess  $\alpha 10$  mRNA, alluding to the presence of Vc1.1-high sensitivity  $\alpha 9(+)-\alpha 9(-)$  site/s in the  $(\alpha 9)_3(\alpha 10)_2$  or  $(\alpha 9)_4(\alpha 10)_1$  configurations. By contrast,  $\alpha 9[N154G]\alpha 10$  nAChRs expressed from  $\alpha 9[N154G]:\alpha 10$  ratios of 1:3 or 3:1 showed similar sensitivity to Vc1.1 inhibition. However, compared to the wild-type receptors, the potency of Vc1.1 inhibition at  $\alpha 9[N154G]\alpha 10$  nAChRs expressed from  $\alpha 9:\alpha 10$  mRNA injection at ratios of 3:1 was decreased ~23-fold whereas at 1:3 mRNA ratio the potency of Vc1.1 was reduced 3-fold. Although the  $\alpha 9$  subunit (+) component N154 residue is postulated to be critical for Vc1.1 high affinity binding at the  $\alpha 9(+)-\alpha 9(-)$  interface, it should be noted that despite the lack of such interface at the 1:3 ratio, Vc1.1 inhibition is affected. The 3-fold reduction of Vc1.1 potency might be explained either by the conformational effects of the mutation to the  $\alpha 10(+)\alpha 9(-)$  binding site or by the

removal of the  $\alpha 9$  N154 at the  $\alpha 9(+)\alpha 10(-)$  binding site.

In contrast to Vc1.1, varying the  $\alpha 9:\alpha 10$  mRNA ratios resulted in minor sensitivity differences of both  $\alpha 9\alpha 10$  and  $\alpha 9[\text{N154G}]\alpha 10$  nAChRs to RgIA# or PeIA inhibition (Figure 4B,C). The  $\alpha 9[\text{N154G}]$  substitution only slightly affected the sensitivity of the mutant receptors to inhibition by both peptides. In our model, the side chain of N154 is not located in close proximity to either PeIA or RgIA#, which may explain their minimal sensitivity difference to either interfaces of  $\alpha 9(+)-\alpha 9(-)$  or  $\alpha 10(+)-\alpha 9(-)$  formed at the different  $\alpha 9\alpha 10$  nAChR stoichiometries. Previous mutagenesis studies suggested that the most favourable binding site of RgIA is at the  $\alpha 10(+)\alpha 9(-)$  interface of  $r(\alpha 9)_2(\alpha 10)_3$  nAChR.<sup>26</sup> Similar to RgIA#, PeIA showed no stoichiometric-dependent inhibition of  $h\alpha 9\alpha 10$  nAChRs, suggesting that both peptides showed no preferable binding to  $\alpha 9(+)-\alpha 9(-)$  versus  $\alpha 10(+)-\alpha 9(-)/\alpha 9(+)-\alpha 10(-)$ .

Vc1.1[D11N] exhibited significantly lower potency than wild-type Vc1.1 at  $h\alpha 9\alpha 10$  nAChRs at both 1:3 and 3:1 ratios with  $\text{IC}_{50}$  values of 17.54  $\mu\text{M}$  (5-fold decrease) and 10.79  $\mu\text{M}$  (14-fold decrease), respectively (Table 1). On the other hand, at  $\alpha 9[\text{N154G}]\alpha 10$  nAChRs, Vc1.1[D11N] and Vc1.1 possess comparable inhibitory activity at both ratios. The substantially reduced inhibitory activity of Vc1.1[D11N] compared to the wild-type Vc1.1 might be due to the removal of R81-D11 charge interaction at both  $\alpha 9(+)\alpha 9(-)$  and  $\alpha 10(+)\alpha 9(-)$  interfaces as well as disruption to the hydrogen bond between N154 and D11 at the  $\alpha 9(+)\alpha 9(-)$  interface (Figure 3A,B). Using thermodynamic mutant cycles,<sup>32</sup> the coupling coefficient ( $\Omega$ ) gave a reciprocal of 28.57 suggesting a relatively strong coupling between Vc1.1 D11 and  $\alpha 9$  N154. Additionally, the coupling energy between Vc1.1 D11 and  $\alpha 9$  N154 was calculated to be 1.95 kcal/mol, comparable to the hydrogen bond energy between N-H and O (~1.9 kcal/mol),<sup>33</sup> further demonstrating the key pairwise interaction between Vc1.1 D11 and  $\alpha 9$  N154.

Vc1.1[PeIA] analogue has residues S9–L15 of PeIA grafted to the corresponding region of Vc1.1 (Figure 2) and similar to Vc1.1[D11N], Vc1.1[PeIA] has an asparagine residue at position 11. Vc1.1[PeIA] exhibited a similar degree of inhibition at both  $h\alpha 9\alpha 10$  and  $h\alpha 9[\text{N154G}]\alpha 10$  nAChRs

expressed from mRNA injection ratios of 1:3 and 3:1. Similar to Vc1.1[D11N], Vc1.1[PeIA] had reduced inhibitory activity at  $\alpha 9\alpha 10$  nAChRs compared to Vc1.1 with the potency decreased  $\sim 4$  fold and  $\sim 12$ -fold at 1:3 and 3:1 ratios, respectively. Thus, PeIA second loop grafting to Vc1.1 resulted in a greater decrease in potency at the  $\alpha 9\alpha 10$  nAChR expressed from high  $\alpha 9:\alpha 10$  mRNA ratio. Furthermore, Vc1.1[PeIA] and Vc1.1 possess comparable  $IC_{50}$  at  $\alpha 9$ [N154G] $\alpha 10$  nAChR (at 1:3 and 3:1 ratios). The insensitivity of Vc1.1[PeIA] to  $\alpha 9\alpha 10$  or  $\alpha 9$ [N154G] $\alpha 10$  nAChRs expressed with excess  $\alpha 9$  (or  $\alpha 9$ [N154G]) or  $\alpha 10$  mRNAs resulted from the replacement of the residues, especially D11 at the second loop of Vc1.1. As shown in PeIA- $\alpha 9(+)\alpha 9(-)$  model (Figure 3F), residues from the second loop of PeIA form few contacts with the side chain of  $\alpha 9$  N154. Thus, similar to Vc1.1[D11N], Vc1.1[PeIA] shows minor activity differences between  $\alpha 9\alpha 10$  nAChRs expressed from mRNA injection ratios of 1:3 and 3:1.

The decreased binding affinity of Vc1.1[D11N] and Vc1.1[PeIA] at the  $\alpha 9\alpha 10$  nAChRs from 1:3, and 3:1 mRNA ratios is unlikely due to the peptide conformational changes. In MD simulations, the backbone RMSD for Vc1.1[D11N] and Vc1.1[PeIA] are comparable to Vc1.1 suggesting that the conformation of both Vc1.1 analogues is similar to the wild-type peptide (Figure S5). In addition, the circular dichroism profiles of Vc1.1[D11N] and Vc1.1[PeIA] were similar to the wild-type peptides, Vc1.1 and PeIA, further suggesting that sequence mutations and sequence replacement resulted in minimal conformation perturbation (Figure S6).

## **Conclusion**

Our computational modelling in combination with mutagenesis studies support that the stoichiometry of the  $\alpha 9\alpha 10$  nAChR expressed with  $\alpha 9:\alpha 10$  mRNA injection ratios of 1:3 and 3:1 could correspond to  $(\alpha 9)_2(\alpha 10)_3$  and  $(\alpha 9)_3(\alpha 10)_2/(\alpha 9)_4(\alpha 10)_1$  nAChRs, respectively. The  $(\alpha 9)_3(\alpha 10)_2/(\alpha 9)_4(\alpha 10)_1$  nAChR arrangement uniquely contains an  $\alpha 9(+)-\alpha 9(-)$  interface binding site that is responsible for the higher sensitivity of the receptor to Vc1.1 than that of the  $(\alpha 9)_2(\alpha 10)_3$  nAChR. The non-conserved residue at position 154 of both  $\alpha 9$  and  $\alpha 10$  subunits essentially differentiates the binding affinity of Vc1.1 to  $\alpha 9(+)-\alpha 9(-)$  and  $\alpha 10(+)-\alpha 9(-)$  interfaces. The

hydrogen bond formed between the side chains of  $\alpha 9$  N154 and Vc1.1 D11 confers the specificity of Vc1.1 at  $\alpha 9(+)-\alpha 9(-)$  interface, and is responsible for the higher sensitivity of the  $\alpha 9\alpha 10$  nAChR expressed with higher abundance of the  $\alpha 9$  mRNA to Vc1.1. The  $\alpha 10(+)-\alpha 9(-)$  interface is the most favourable Vc1.1 binding site at  $(\alpha 9)_2(\alpha 10)_3$  nAChR whereas at  $(\alpha 9)_3(\alpha 10)_2/(\alpha 9)_4(\alpha 10)_1$  nAChR, the interface of  $\alpha 9(+)-\alpha 9(-)$  rather than  $\alpha 10(+)-\alpha 9(-)$  interface has higher affinity for Vc1.1. Despite sharing the same disulfide-bond framework with Vc1.1, PeIA demonstrates no obvious stoichiometry-dependent inhibition of  $\alpha 9\alpha 10$  nAChRs and it also does not discriminate between either  $\alpha 9(+)-\alpha 9(-)$  or  $\alpha 10(+)-\alpha 9(-)$  interfaces from our modelling studies. RgIA# exhibits no preferential inhibitory activity at either  $(\alpha 9)_3(\alpha 10)_2$  or  $(\alpha 9)_2(\alpha 10)_3$  nAChRs and residues of RgIA# have no remarkable specific pairwise interactions to the interfaces of  $\alpha 9(+)-\alpha 9(-)$  or  $\alpha 10(+)-\alpha 9(-)$ . However, we cannot exclude the possibility of the  $\alpha 9(+)-\alpha 9(-)$  as an energetically favourable binding site of RgIA# at  $(\alpha 9)_3(\alpha 10)_2$  nAChR. Instead, our study suggests that the sensitivity of both  $\alpha 10(+)-\alpha 9(-)$  and  $\alpha 9(+)-\alpha 9(-)$  interfaces to RgIA# might be comparable. In a previous study, the contribution of  $\alpha 9$  and  $\alpha 10$  subunits to complementary components of the ligand-binding site was proposed to be non-equivalent,<sup>34</sup> whereas we found the contribution of  $\alpha 9$  and  $\alpha 10$  subunits to principal components of the ligand-binding site was non-equivalent for Vc1.1 binding but could be equivalent for the binding of RgIA# and PeIA.

In summary, elucidation of the determinants that confer the selectivity of the  $\alpha$ -conotoxins to varied subunit arrangements of the  $\alpha 9\alpha 10$  nAChR is essential for rational design of novel stoichiometric-selective  $\alpha$ -conotoxin analogues. Whether different stoichiometries of  $\alpha 9\alpha 10$  nAChR exist *in vivo* is yet to be determined. Regardless, stoichiometric-selective inhibitors of  $\alpha 9\alpha 10$  nAChR would be useful neurochemical tools for further elucidating the functional differences between the stoichiometries in native cells. Conversely,  $\alpha 9\alpha 10$  nAChR antagonists that do not discriminate between the stoichiometries, may be suitable candidates to use under conditions where different stoichiometries exist.

## Methods

### *Homology modeling*

Models of Vc1.1 bound  $h(\alpha 9)_2(\alpha 10)_3$  and  $h(\alpha 9)_2(\alpha 10)_3$  nAChRs were built using Modeller (version 9v14), as described previously.<sup>24,30</sup> The extracellular domain sequence of the  $h\alpha 1$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 9$ , and  $\alpha 10$  nAChR subunits were retrieved from the Uniprot database.<sup>35</sup> Both crystal structures of *Aplysia californica* AChBP (acetylcholine binding protein) in complex with  $\alpha$ -conotoxin PnIA[A10L,D14K] (PDB code 2BR8), and the  $h\alpha 9$  nAChR subunit extracellular domain (PDB code 4D01) from the Protein Data Bank, were used as templates to build 200 models of each  $\alpha$ -conotoxin-nAChR complexes.<sup>36,37</sup> Models with the lowest DOPE score were selected for further structural refinement using molecular dynamics (MD) simulations. The structures of Vc1.1 analogues, Vc1.1[D11N] and Vc1.1[PeIA], were generated using Modeller based on the NMR structure of Vc1.1(PDB code 2H8S).<sup>38</sup>

### *Molecular dynamic simulations*

The protonation states of His, Asp and Glu residues at the conotoxin/nAChR complexes were predicted using the PropKa 3.1 method.<sup>39</sup> The models were minimized and refined using molecular dynamics (MD) simulations performed with the Amber 16 package and ff14SB force field.<sup>40,41</sup> The receptor complexes were solvated in a truncated octahedral TIP3P water box containing ~10800 water molecules. Sodium ions were added to neutralize the systems. The systems were first minimized with 3,000 steps of steepest descent and then 3,000 steps of conjugate gradient with the solute restrained to their position by a harmonic force of  $100 \text{ kcal/mol} \cdot \text{\AA}^2$ . A second minimization was then performed but with all position restraints withdrawn. The systems were then gradually heated up from 50 to 300 K in the NVT ensemble over 100 ps with the solute restrained to their position using a  $5 \text{ kcal/mol} \cdot \text{\AA}^2$  harmonic force potential. MD simulations were then carried out in the NPT ensemble, and the position restraints were gradually removed over 100 ps. The production runs were conducted over 50 ns simulation time with pressure coupling set at 1 atm and a constant temperature of 300 K. The MD simulations used a time step of 2 fs and, all bonds involving

hydrogen atoms were maintained to their standard length using the SHAKE algorithm.<sup>42</sup> The particle-mesh Ewald (PME) method was used to model long-range electrostatic interactions.<sup>43</sup> MD trajectories were analyzed using VMD (<http://www.ks.uiuc.edu/>) and molecules were drawn using PyMol (Schrödinger, LLC).

For validation of the Vc1.1 analogue stability, 100 ns repeated MD simulations were performed on Vc1.1[N9D] and Vc1.1[PeIA] systems respectively, using the same method as described above.

#### *Binding energy decomposition*

To quantify the binding energy contribution of these key residues in the ligand binding site, binding energy decomposition was carried out using the MMPBSA.py script in AMBER16.<sup>44</sup> The binding free energy ( $\Delta G_{\text{binding}}$ ) values were calculated using the following equation:

$$\Delta G_{\text{binding}} = G_{\text{complex}} - G_{\text{ligand}} - G_{\text{receptor}} \quad (1)$$

where the binding free energy ( $\Delta G_{\text{binding}}$ ) was determined using MMGB/SA method.<sup>45</sup> This method was identified in our previous study as being slightly more efficient for ranking mutational energies based on homology models than the MMPB/SA method. Details of the MMGB/SA method on peptide binding energy calculation was described in our previous modelling studies.<sup>30</sup>

#### *Thermodynamic mutant cycles*

The coupling coefficient ( $\Omega$ ) and coupling energy ( $\Delta G_{\text{couple}}$ ) was determined using equations (2) and (3) respectively:

$$\Omega = K_i \text{ of wild-type toxin at wild-type channel} \cdot K_i \text{ of mutant toxin at mutant channel} / K_i \text{ of wild-type toxin at mutant channel} \cdot K_i \text{ of mutant toxin at wild-type channel} \quad (2) \quad ^{32}$$

where  $K_i$  = equilibrium inhibition constant ( $IC_{50}$ )

$$\Delta G_{\text{couple}} = -RT \ln(\Omega) \quad (3)$$

where R = gas constant, T = temperature

#### *Peptide synthesis*

Procedures for solid peptide synthesis of Vc1.1 analogues were nearly the same as described previously.<sup>24</sup> Briefly, Vc1.1[D11N] and Vc1.1[PeIA] were assembled on rink amide

methylbenzhydrylamine resin (Novabiochem) using solid-phase peptide synthesis with a neutralization/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation procedure for Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. Cleavage was achieved by treatment with 88:5:5:2 ratio of trifluoroacetic acid (TFA), phenol, water and triisopropylsilane as scavengers, at room temperature (20–25 °C) for 2 h. TFA was evaporated at low pressure in a rotary evaporator. Peptides were precipitated with ice-cold ether, filtered, dissolved in 50% buffer A/B (buffer A consists of 99.95% H<sub>2</sub>O/ 0.05% TFA and buffer B consists of 90% CH<sub>3</sub>CN/10% H<sub>2</sub>O/0.045% TFA), and lyophilized. Crude peptides were purified by RP-HPLC on a Phenomenex C<sub>18</sub> column, and its molecular mass was confirmed using electrospray mass spectrometry before they were pooled and lyophilized for oxidation. The four cystines in the peptides were selectively oxidized in two steps. In the first step the non-protected cystines were oxidized in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> (pH 8–8.5) at a concentration of 0.5 mg/ml, and stirred at room temperature overnight. In the second step, the Ac<sub>2</sub>S-protected cystines were oxidized by dissolving the peptides in iodine solution filled at concentration of 1 mg/ml, and stirred for 30 min. Ascorbic acid was then added to stop the oxidizing reaction and the solution was stirred again until no colour was visible. After two rounds of oxidation, peptides were purified by RP-HPLC and their mass (Figure S7) and purity (Figure S8) were validated using electrospray-mass spectrometry (MS) and analytical RP-HPLC, respectively.

#### *Circular dichroism (CD) study*

CD spectra were performed on Jasco J-810 spectropolarimeter over the wavelength range of 250–190 nm using a 1.0 mm path length cell, a bandwidth of 1.0 nm, a response time of 2 s, and averaging over three scans. Spectra were recorded at room temperature under nitrogen atmosphere. Peptides were dissolved in buffer A and buffer B. The concentration of Vc1.1, Vc1.1[D11N], Vc1.1[PeIA], and PeIA was 0.28 mM, 0.28 mM, 0.29 mM and 0.30 mM, respectively. The spectra are expressed as molar ellipticity ( $[\theta]$ · $[\theta] = 1000 \cdot \text{mdeg}/(l \cdot c)$  where, mdeg is the raw CD data, *c* is the peptide molar concentration (mM), and *l* is cell path length (mm)).

#### *PCR site-directed mutagenesis*



N154G mutation to the  $\alpha 9$  nAChR subunit in plasmid pT7TS was done using PrimeSTAR Max kit (Takara Bio, Kusatsu, Shiga, Japan). Mutation was confirmed by DNA sequencing (Australian Genome Research Facility, Melbourne, VIC, Australia).

#### *In vitro cRNA synthesis*

Plasmid pT7TS constructs of  $\alpha 9$  and  $\alpha 10$  nAChR subunits were linearized with *XbaI* restriction enzyme (NEB, Ipswich, MA) for *in vitro* cRNA transcription using T7 mMessage mMachine transcription kits (AMBION, Foster City, CA, USA).

#### *Oocyte preparation and microinjection.*

All procedures were approved by the University of Sydney Animal Ethics Committee (project number 2016/970). Stage V-VI oocytes (Dumont's classification; 1200-1300  $\mu\text{m}$  diameter) were obtained from *Xenopus laevis*, defolliculated with 1.5 mg/ml collagenase Type II (Worthington Biochemical Corp., Lakewood, NJ) at room temperature for 1-2 h in OR-2 solution containing (in mM) 82.5 NaCl, 2 KCl, 1  $\text{MgCl}_2$  and 5 HEPES at pH 7.4. Oocytes were injected with 35 ng cRNA for  $\alpha 9\alpha 10$  nAChR (concentration confirmed spectrophotometrically and by gel electrophoresis) at  $\alpha 9:\alpha 10$  subunit mRNA ratios of 1:1, 1:3 and 3:1, using glass pipettes pulled from glass capillaries (3-000-203 GX, Drummond Scientific Co., Broomall, PA, USA). Oocytes were incubated at 18 °C in sterile ND96 solution composed of (in mM) 96 NaCl, 2 KCl, 1  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$  and 5 HEPES at pH 7.4, supplemented with 5% FBS, 50 mg/L gentamicin (GIBCO, Grand Island, NY, USA) and 10000 U/mL penicillin-streptomycin (GIBCO, Grand Island, NY, USA).

#### *Oocyte two-electrode voltage clamp recording and data analysis.*

Electrophysiological recordings were carried out 2–5 days post cRNA microinjection. Two-electrode voltage clamp recordings of *X. laevis* oocytes expressing human nAChRs were performed at room temperature (21–24 °C) using a GeneClamp 500B amplifier and pClamp9 software interface (Molecular Devices, Sunnyvale, CA, USA) at a holding potential  $-80$  mV. Voltage-recording and current-injecting electrodes were pulled from GC150T-7.5 borosilicate glass (Harvard Apparatus, Holliston, MA) and filled with 3 M KCl giving resistances of 0.3–1  $\text{M}\Omega$ .

Oocytes were incubated in 100  $\mu$ M BAPTA-AM ~3 h before recording and perfused with ND115 solution containing (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl<sub>2</sub>, and 10 HEPES at pH 7.4 using a continuous Legato 270 push/pull syringe pump perfusion system (KD Scientific, Holliston, MA, USA) at a rate of 2 mL/min in an OPC-1 perfusion chamber of < 20  $\mu$ L volume (Automate Scientific, Berkeley, CA, USA). Due to the Ca<sup>2+</sup> permeability of  $\alpha 9\alpha 10$  nAChRs, BAPTA-AM incubation was carried out to prevent the activation of *X. laevis* oocyte endogenous Ca<sup>2+</sup>-activated chloride channels.

Initially, oocytes were briefly washed with ND115 solution followed by 3 applications of acetylcholine (ACh) at a half-maximal excitatory ACh concentration (EC<sub>50</sub>) for  $\alpha 9\alpha 10$  nAChRs. Washout with bath solution was done for 3 min between ACh applications. Oocytes were incubated with peptides for 5 min with the perfusion system turned off, followed by co-application of ACh and peptide with flowing bath solution. All peptide solutions were prepared in ND115 + 0.1 % bovine serum albumin. Peak current amplitudes before (ACh alone) and after (ACh + peptide) peptide incubation were measured using Clampfit version 10.7.0.3 software (Molecular Devices, Sunnyvale, CA, USA), where the ratio of ACh + peptide-evoked current amplitude to ACh alone-evoked current amplitude was used to assess the activity of the peptides at  $\alpha 9\alpha 10$  nAChRs. All electrophysiological data were pooled (n = 3-16) and represent means  $\pm$  standard error of the mean (SEM). Data analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). ACh EC<sub>50</sub> and  $\alpha$ -conotoxin IC<sub>50</sub> values were determined from concentration-response relationships fitted to a non-linear regression function and reported with error of the fit. Data sets were compared using Tukey's test. Differences were regarded statistically significant when  $p < 0.05$ .

### Supporting Information

Activity of the  $\alpha$ -conotoxins at the human  $\alpha 9\alpha 10$  nAChRs is shown in Figure S1. Internal backbone H-bond between PeIA residues H5 and C8 is shown in Figure S2. Side chain energetic contribution to the  $\alpha$ -conotoxin binding affinity is shown in Figure S3. Activities of the  $\alpha$ -conotoxins at human

$\alpha 9\alpha 10$  and  $\alpha 9$ [N154G] $\alpha 10$  nAChRs expressed from varied  $\alpha 9\alpha 10$  mRNA ratios are shown in Figure S4. Structural comparison of wild-type Vc1.1 and analogues Vc1.1[D11N] and Vc1.1[PeIA] from MD simulations are shown in Figure S5. Circular dichroism spectra of Vc1.1, Vc1.1[D11N], Vc1.1[PeIA] and PeIA are shown in Figure S6. MS and analytical HPLC spectra of Vc1.1, Vc1.1[D11N], Vc1.1[PeIA], PeIA and RgIA# are shown in Figure S7 and Figure S8, respectively.

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<sup>†</sup>(R.Y, H-S.T.) These authors contributed equally to this work. R.Y. performed computational modeling, analyzed the data, and contributed to writing the paper; H-S.T. conducted the electrophysiological experiments, analyzed the data and contributed to writing the paper; N.T. J. S. and T. J. conducted peptide synthesis; D.J.A., R.Y. and H-S.T. conceived the idea of the project, provided the financial support and resources, and contributed to writing the paper.

### **Notes**

The authors declare no competing financial interest.

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### **Abbreviations Used**

nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein; HBTU, neutralization/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MBHA; rink amide methylbenzhydrylamine; MD, molecular dynamics; RP-HPLC, reversed-phase HPLC;

Vc1.1,  $\alpha$ -conotoxin Vc1.1; PeIA,  $\alpha$ -conotoxin PeIA; RgIA#,  $\alpha$ -conotoxin RgIA with the R13 truncated and C-termini amidation; Vc1.1[D11N],  $\alpha$ -conotoxin Vc1.1 [D11N] mutant; Vc1.1[PeIA],  $\alpha$ -conotoxin Vc1.1 grafted with residues from the second loop of PeIA; RMSD, root mean square deviation; EC<sub>50</sub>, half-maximal excitatory concentration; IC<sub>50</sub>, half-maximal inhibitory concentration.

### **Homology models**

Authors will release the atomic coordinates and experimental data upon article publication.

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**Table 1. Inhibition of h $\alpha$ 9 $\alpha$ 10 and h $\alpha$ 9[N154] $\alpha$ 10 expressed from  $\alpha$ 9/ $\alpha$ 9[N154G]: $\alpha$ 10 mRNA injection ratios of 1:3 and 3:1 by Vc1.1 and analogues.**

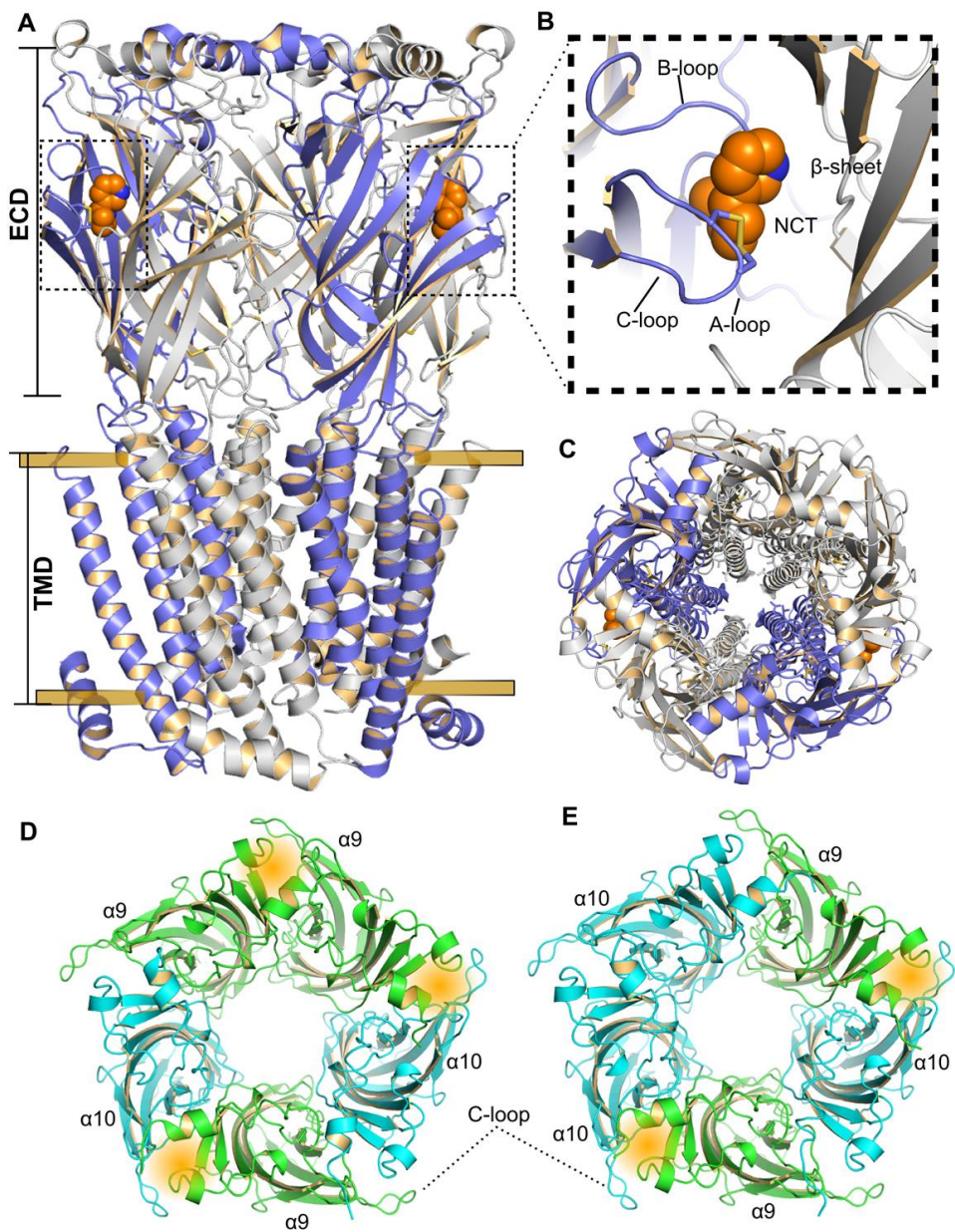
Receptor	$\alpha$ 9: $\alpha$ 10	ACh ( $\mu$ M)	Vc1.1 ( $\mu$ M)	Vc1.1[D11N] ( $\mu$ M)	Vc1.1[PeIA] ( $\mu$ M)
h $\alpha$ 9 $\alpha$ 10	1:3	47.42 $\pm$ 5.77	3.57 $\pm$ 0.31	17.54 $\pm$ 2.11	13.23 $\pm$ 1.80
	3:1	40.00 $\pm$ 5.98	0.75 $\pm$ 0.09	10.79 $\pm$ 1.14	9.30 $\pm$ 0.86
h $\alpha$ 9[N154G] $\alpha$ 10	1:3	13.12 $\pm$ 0.93	11.25 $\pm$ 1.50	10.24 $\pm$ 0.69	12.96 $\pm$ 1.89
	3:1	34.28 $\pm$ 3.99	17.02 $\pm$ 3.17	8.59 $\pm$ 0.77	15.2 $\pm$ 1.34

EC<sub>50</sub> values (mean  $\pm$  error of the fit, n = 3-4) obtained from the fit of the ACh concentration-response relationship for h $\alpha$ 9 $\alpha$ 10 and h $\alpha$ 9[N154G] $\alpha$ 10 nAChRs expressed from  $\alpha$ 9/ $\alpha$ 9[N154G]: $\alpha$ 10 mRNA injection ratios of 1:3 and 3:1

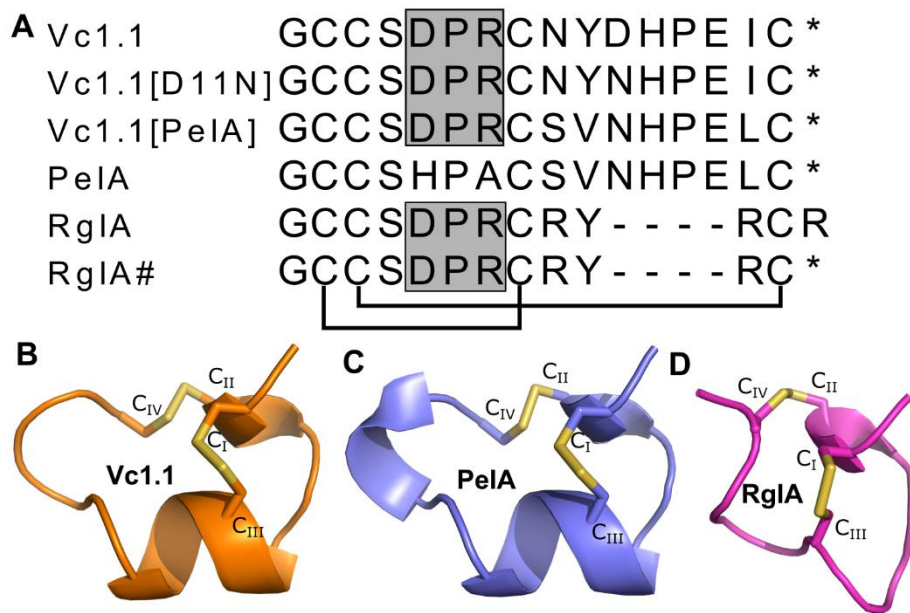
IC<sub>50</sub> values (mean  $\pm$  error of the fit, n = 3-16) obtained from the fit of the concentration-response relationships for the inhibition of h $\alpha$ 9 $\alpha$ 10 and h $\alpha$ 9[N154G] $\alpha$ 10 nAChRs expressed from  $\alpha$ 9/ $\alpha$ 9[N154G]: $\alpha$ 10 mRNA injection ratios of 1:3 and 3:1 by Vc1.1, Vc1.1[D11N], and Vc1.1[PeIA].



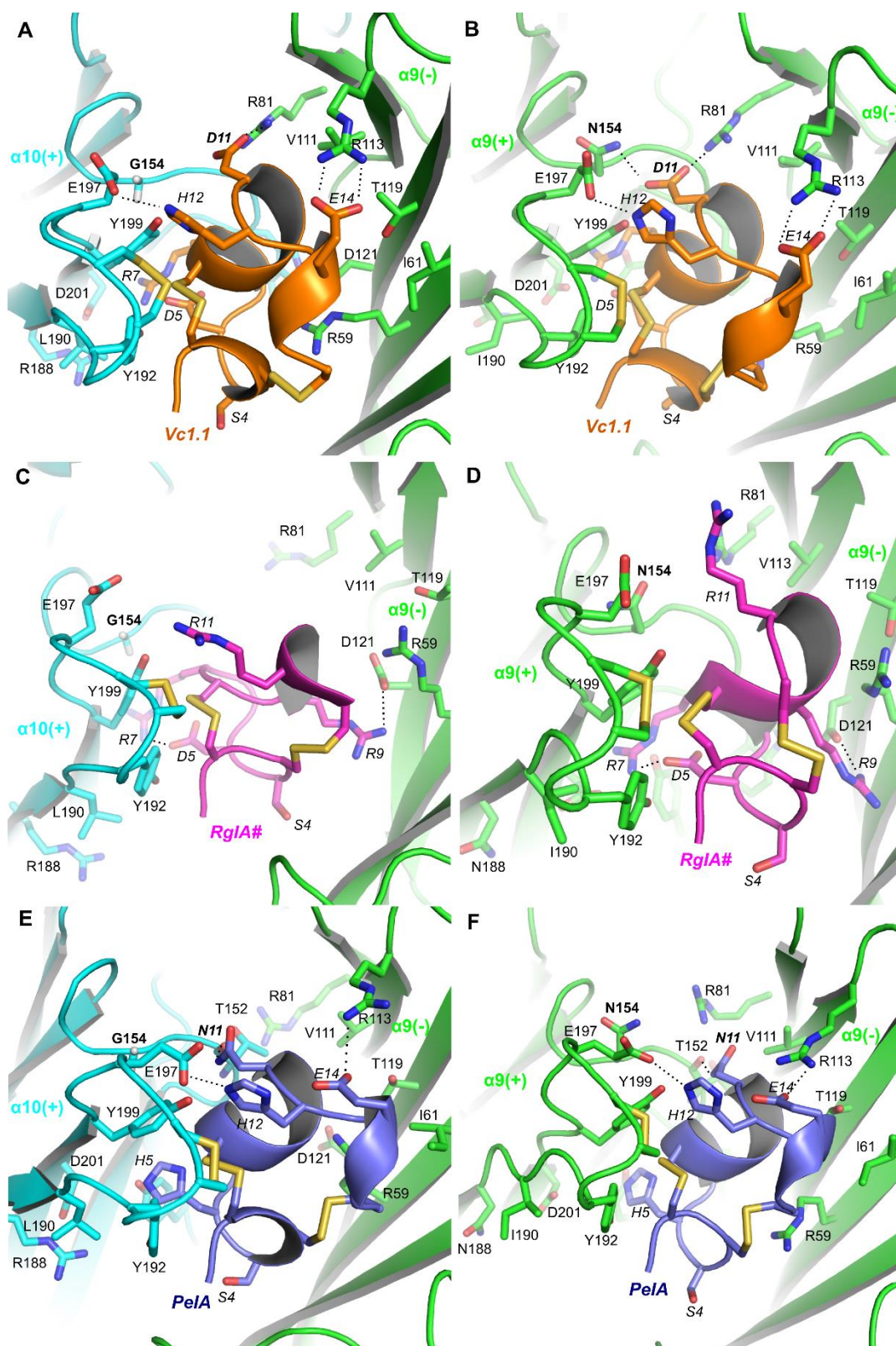
Figures



**Figure 1. Structures of the nAChRs.** (A) Side view of the human  $\alpha 4\beta 2$  nAChR crystal structure showing the extracellular (ECD) and transmembrane (TMD) domains. Two nicotine molecules (NCT, orange) are bound to the ECD binding site (dashed frame) (PDB code: 5KXI). The  $\alpha 4$  and  $\beta 2$  subunits are colored blue and grey, respectively. (B) The ligand binding site magnified (dashed frame) comprising of loops A, B and C of the  $\alpha 4$  subunit (principal component (+)) and  $\beta$ -sheet of the  $\beta 4$  subunit (complementary component (—)); (C) Top view of the human  $\alpha 4\beta 2$  nAChR; (D,E) Top view of the  $(\alpha 9)_2(\alpha 10)_3$  nAChR and  $(\alpha 9)_3(\alpha 10)_2$  nAChR homology models, respectively. The  $\alpha$ -conotoxin binding site is located at the cleft between the  $\alpha 10(+)$  and  $\alpha 9(—)$  components or  $\alpha 9(+)$  and  $\alpha 9(—)$  components (orange cloud). The (+) component contributes the C-loop to the binding site, and the (—) component contributes the  $\beta$ -sheet to the binding pocket. These models were built using multiple templates, including the crystal structures of *Aplysia californica* AChBP (PDB code: 2BR8) and the human  $\alpha 9$  nAChR subunit ECD (PDB code: 4UY2).



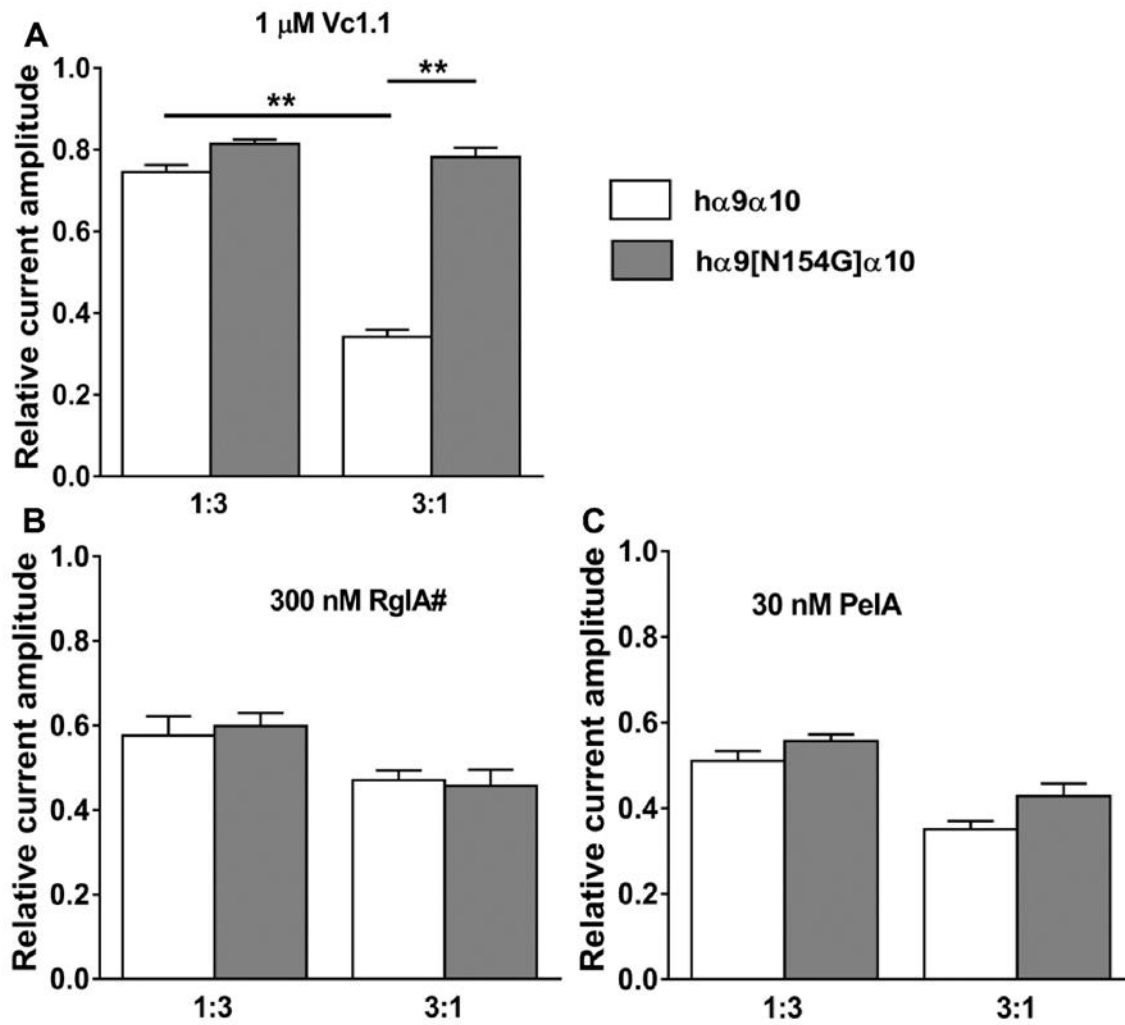
**Figure 2: Sequence alignment and structures of the  $\alpha$ -conotoxins.** (A) Sequence alignment of the  $\alpha$ -conotoxin Vc1.1, PeIA, RgIA# and their analogues. There are two disulphide bonds (black lines) linking C<sub>I</sub> and C<sub>III</sub>, and C<sub>II</sub> and C<sub>IV</sub>, respectively. The DPR motif is highlighted using the grey background. Vc1.1 and PeIA possess a 4/7 disulphide framework, and RgIA has a 4/3 disulphide framework. \* represents the amide group of the C-termini. (B) and (D) NMR structures of Vc1.1 (PDB code: 2H8S) and RgIA (PDB code: 2JUT), and (C) crystal structure of PeIA (PDB code: 5JME).



**Figure 3. Binding of the  $\alpha$ -conotoxins at the human  $\alpha 10(+)$ - $\alpha 9(-)$  and  $\alpha 9(+)$ - $\alpha 9(-)$  interfaces.**

(A,B), (C,D), and (E,F)  $\alpha$ -Conotoxins Vc1.1, RgIA# and PeIA bound to the interfaces of  $\alpha 10(+)$ - $\alpha 9(-)$  (left panels) and  $\alpha 9(+)$ - $\alpha 9(-)$  (right panels), respectively. The  $\alpha 10$  and  $\alpha 9$  subunits are shown in cyan and green, respectively. Dashed lines indicate hydrogen bonds formed between the  $\alpha$ -conotoxins and  $\alpha 9\alpha 10$  nAChR.





**Figure 4. Activity of the  $\alpha$ -conotoxins at human  $\alpha$ 9 $\alpha$ 10 and  $\alpha$ 9[N154G] $\alpha$ 10 nAChRs expressed from varied  $\alpha$ 9: $\alpha$ 10 mRNA ratios.** Bar graphs of relative ACh-evoked current amplitude mediated by h $\alpha$ 9 $\alpha$ 10 and h $\alpha$ 9[N154G] $\alpha$ 10 nAChRs in the presence of (A) 1  $\mu$ M Vc1.1, (B) 300 nM RgIA#, and (C) 30 nM PeIA. Whole-cell h $\alpha$ 9 $\alpha$ 10 nAChR-mediated currents at 1:3 and 3:1 ratios were activated by 50  $\mu$ M and 30  $\mu$ M ACh, respectively, and h $\alpha$ 9[N154G] $\alpha$ 10 nAChR-mediated currents at 1:3 and 3:1 ratios were activated by 20  $\mu$ M and 30  $\mu$ M ACh, respectively (close to their respective EC<sub>50</sub> values). \*\* p < 0.0001