

**SUPPORTING INFORMATION****A conserved arginine with non-conserved function is a key determinant of agonist selectivity in  $\alpha 7$  nicotinic acetylcholine receptors**

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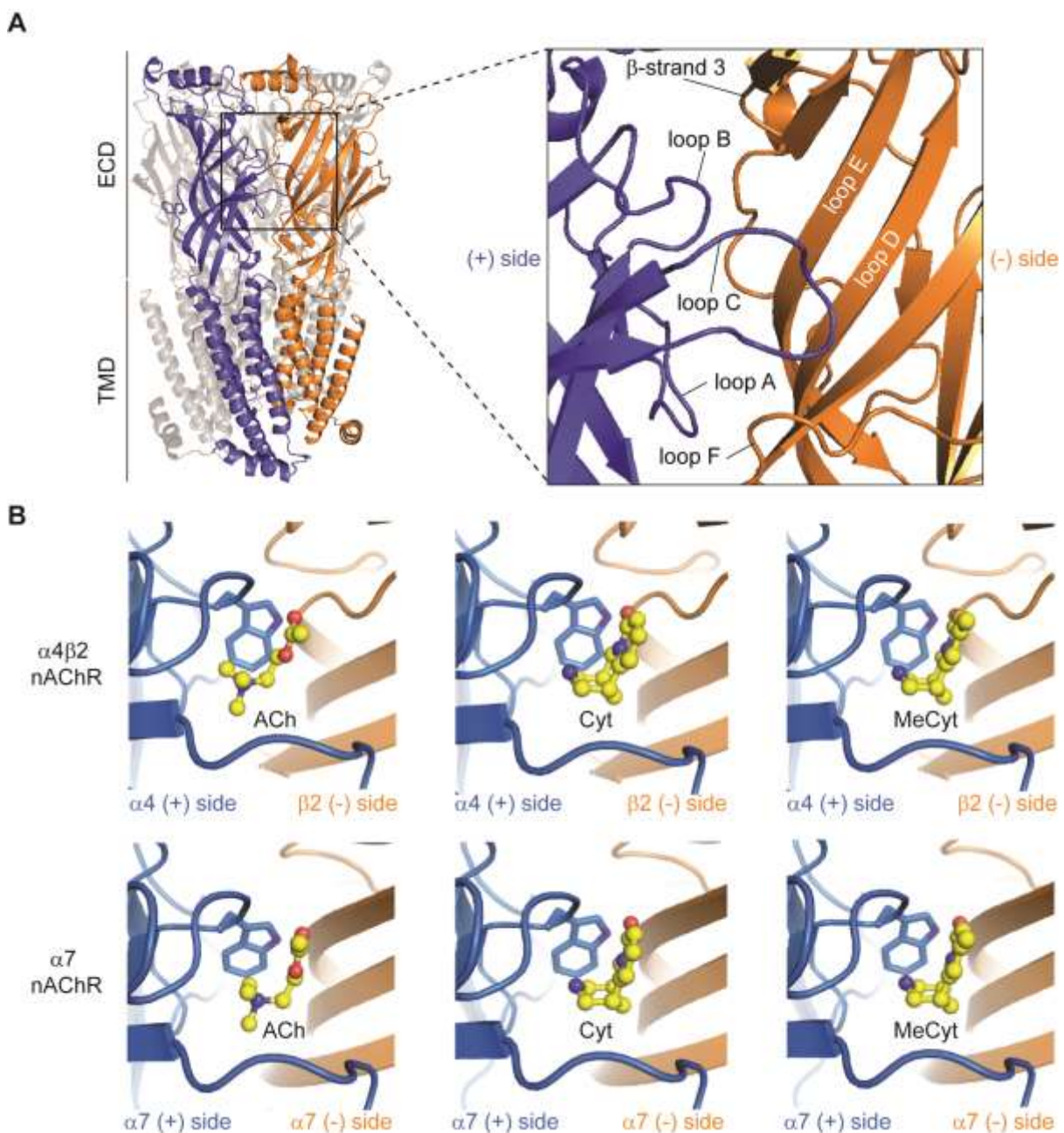
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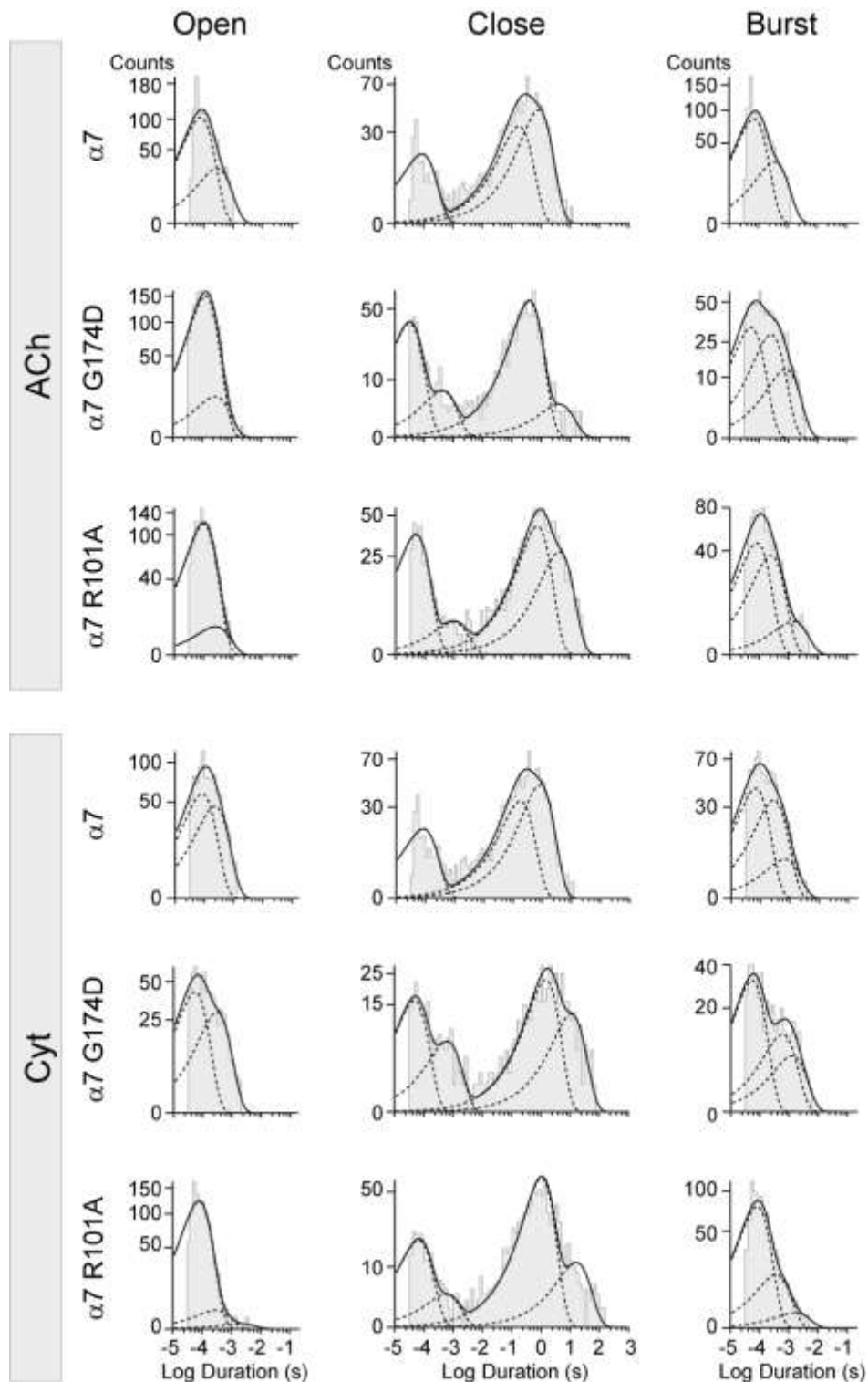
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## 1. Supporting Figures

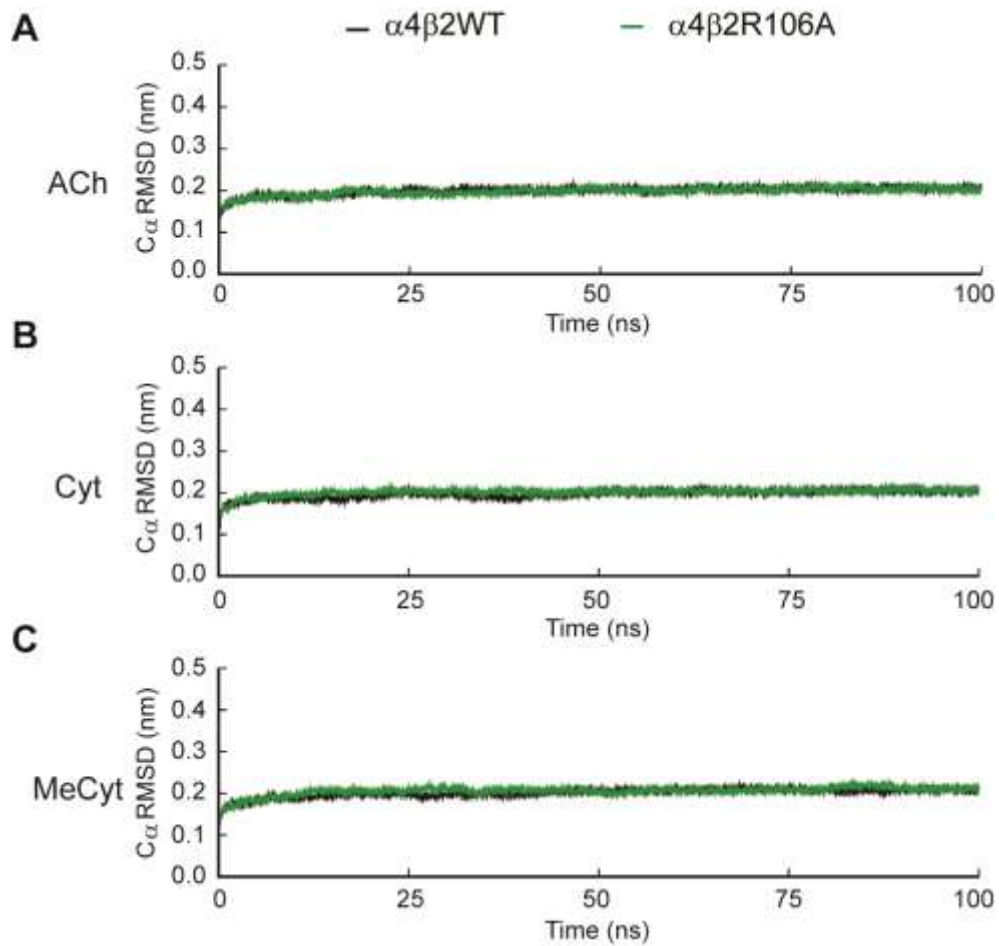
**Figure S1. A)** The nAChR structure and the agonist binding pocket. The structure shown here corresponds to the crystal structure of the  $\alpha 4\beta 2$  nAChR (PDB code: 5KXI) (Perez-Morales et al., 2016). The principal subunit ( $\alpha 4$ ) is shown in blue, and the complementary subunit ( $\beta 2$ ) is shown in orange. The structure includes the transmembrane domain and the ECD with the inset showing a close-up view of the agonist binding pocket with loops A-C in the principal subunit and loops D-F in the complementary subunit highlighted. Note that although historically known as loops, loops E and D are indeed  $\beta 3$ -strands. **B)** Binding mode of ACh, cytosine and 10-methylcytosine in  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs. Agonists are represented as balls-and-sticks, and the tryptophan residue (TrpB) that provides the anchor point for the agonist in the binding site is shown with sticks.



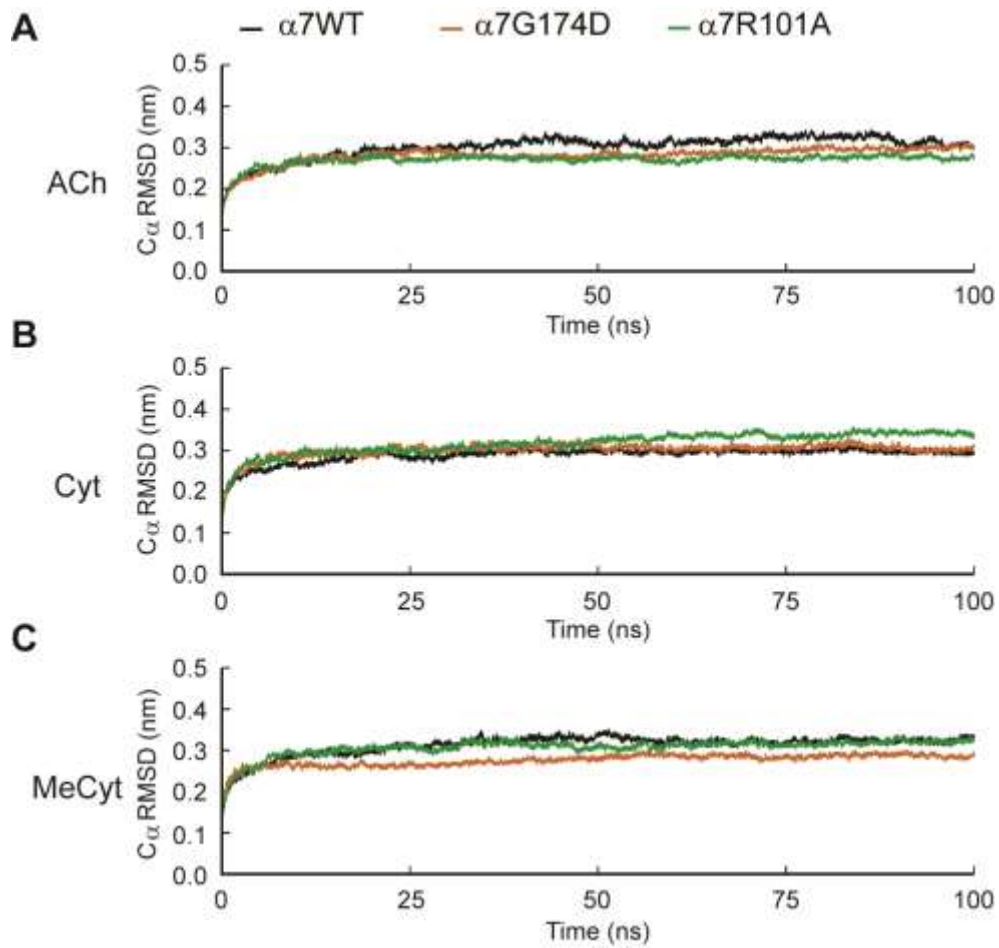
**Figure S2.** Representative dwell time histograms of single-channel recordings from  $\alpha 7$ ,  $\alpha 7$ G174D and  $\alpha 7$ R101A activated by Ach (top) or cytisine (bottom). Dwell times are shown on logarithmic time axes with overlaid fits to the sum of exponentials (solid line, fits; dotted lines, individual components). Burst duration histograms are open time histograms generated by summing open times with flanking closings briefer than a critical time determined from corresponding closed time histograms (see Methods). Membrane potential: -70 mV. Abbreviations: cytisine, Cyt; 10-methylcytisine, MeCyt.



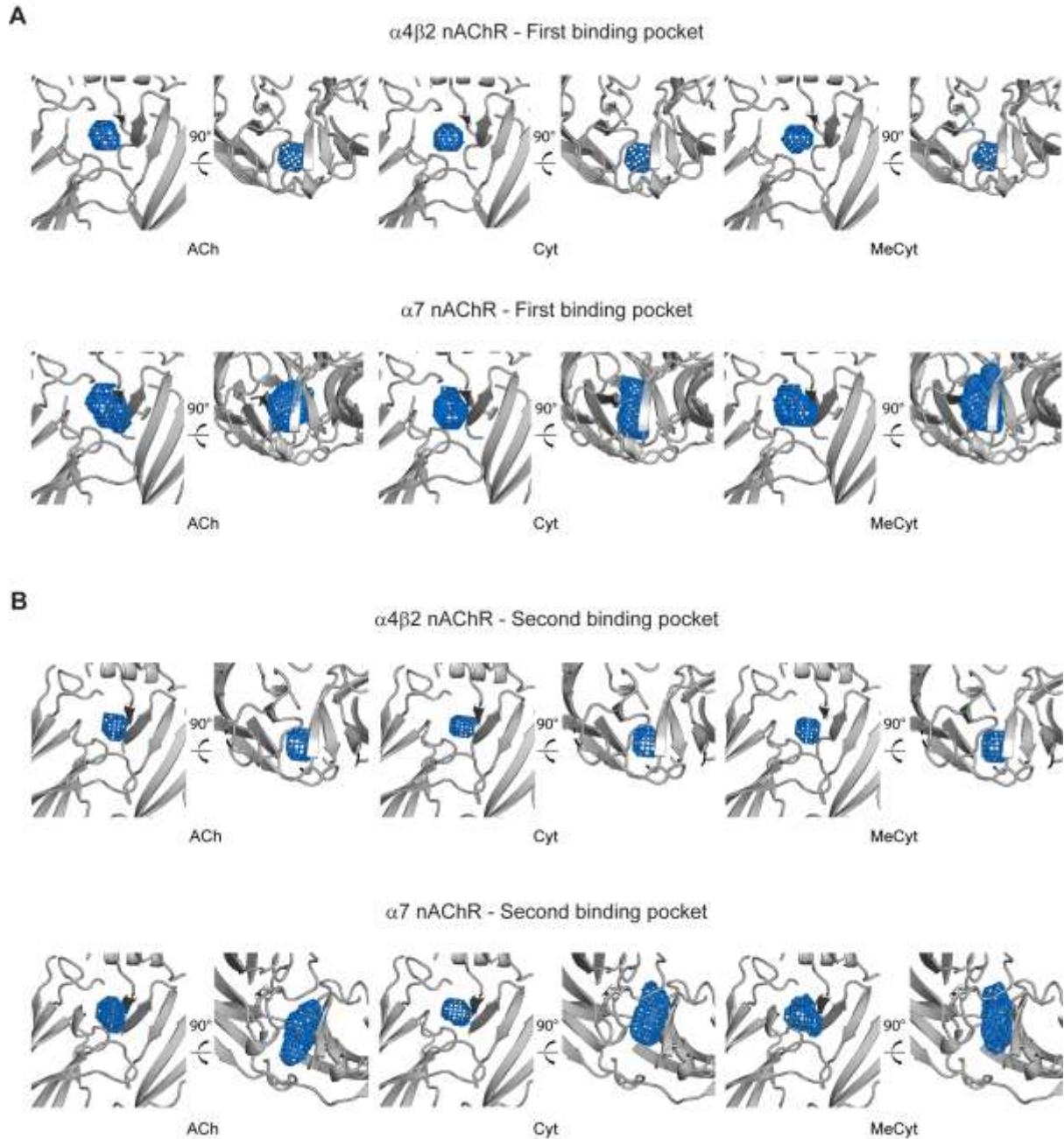
**Figure S3. Temporal evolution of the average  $C_{\alpha}$  RMSD for the  $\alpha 4\beta 2$  systems.** The  $C_{\alpha}$ RMSD was calculated relative to the starting structures, and the averages were obtained over all replicates. Agonist shown are **A)** ACh, **B)** cytosine and **C)** 10-methylcytosine. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



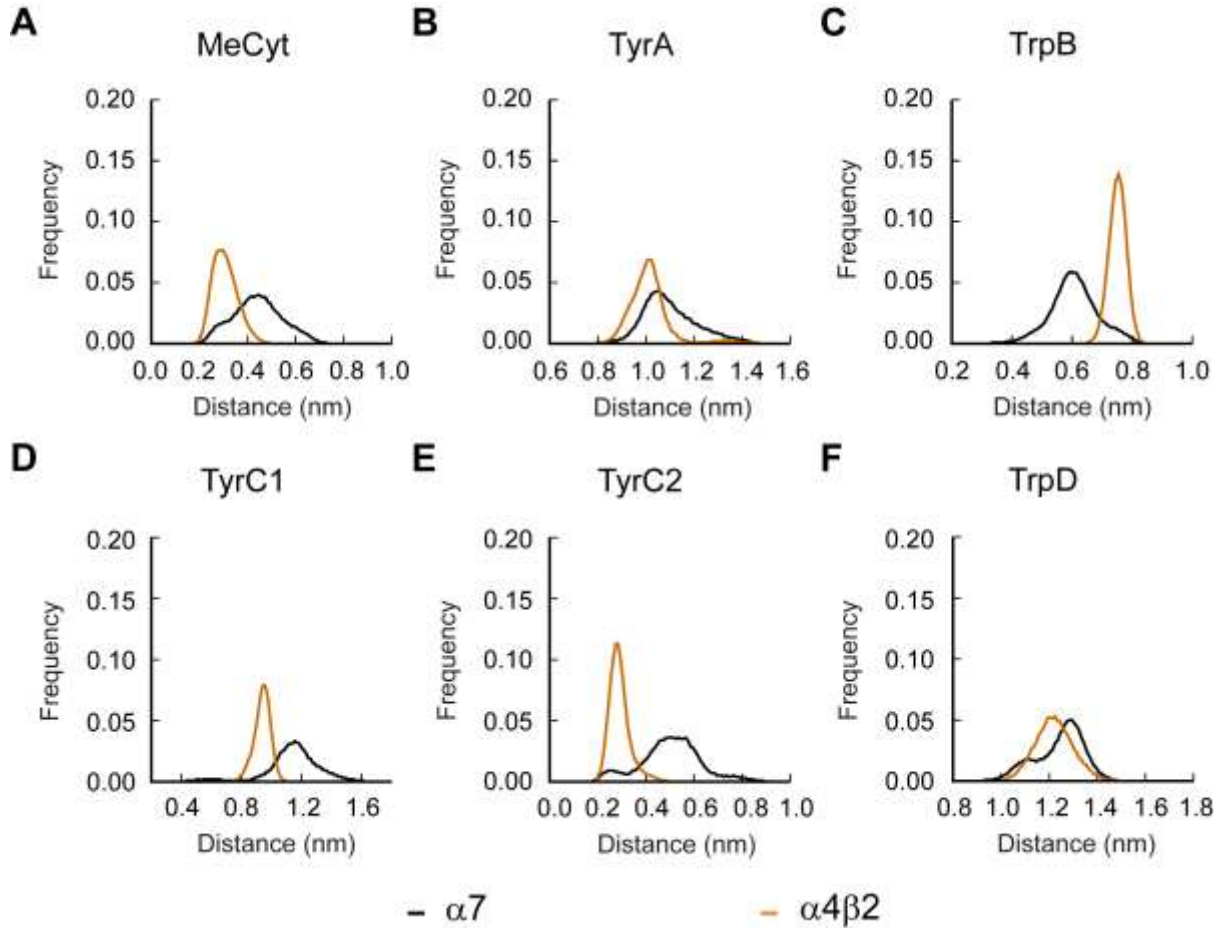
**Figure S4. Temporal evolution of the average  $C_{\alpha}$  RMSD for the  $\alpha 7$  systems.** The  $C_{\alpha}$ RMSD was calculated relative to the starting structures, and the averages were obtained over all replicates. Agonist shown are **A)** ACh, **B)** cytosine and **C)** 10-methylcytosine. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



**Figure S5.** Probability density maps for the side chain of arginine R106 in the  $\beta 2$  subunit and R101 in the  $\alpha 7$  nAChR complexes of the **A)** first and **B)** second of the two non-consecutive agonist binding pockets analysed. The complexes are bound to ACh, cytosine or 10-methylcytosine. No noticeable differences are observed between the maps for the two binding sites. The contours at  $0.00001 \text{ \AA}^{-3}$  for the C $\zeta$  are depicted as a blue mesh. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



**Figure S6.** Distribution of the minimum distance between the conserved arginine in  $\beta$ 3-strand (R101 in  $\alpha$ 7 and R106 in  $\alpha$ 4 $\beta$ 2) and **A**) 10-methylcytosine, **B**) TyrA, **C**) TrpB, **D**) TyrC1, **E**) TyrC2 and **F**) TrpD in the  $\alpha$ 7 (black line) and  $\alpha$ 4 $\beta$ 2 (orange line) systems. The histograms reflect the distances over the two binding pockets. Note that these distance profiles clearly show a distinct pattern of behavior for the  $\beta$ 3-strand arginine between the  $\alpha$ 7 and  $\alpha$ 4 $\beta$ 2 receptors. Abbreviations: 10-methylcytosine, MeCyt.

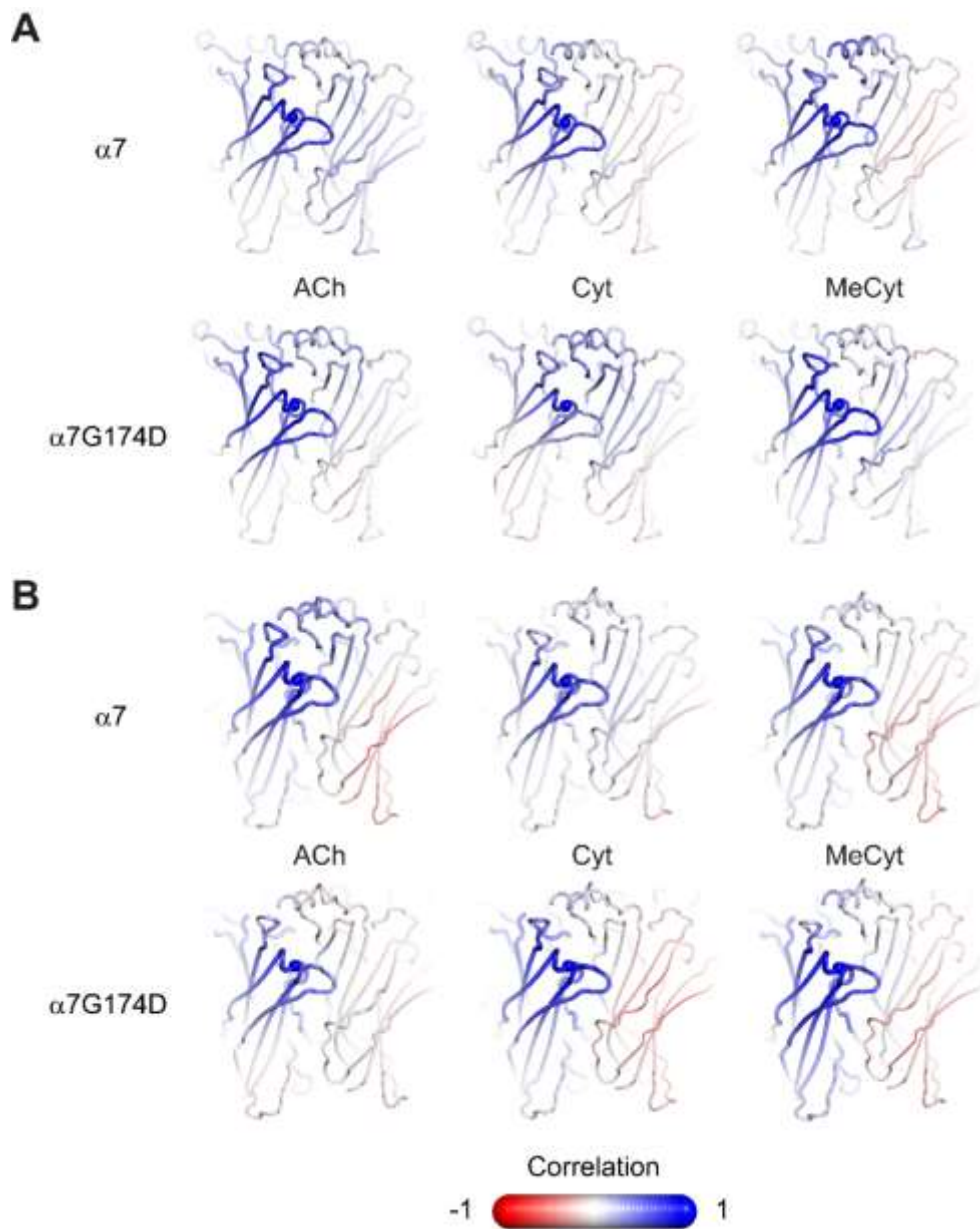




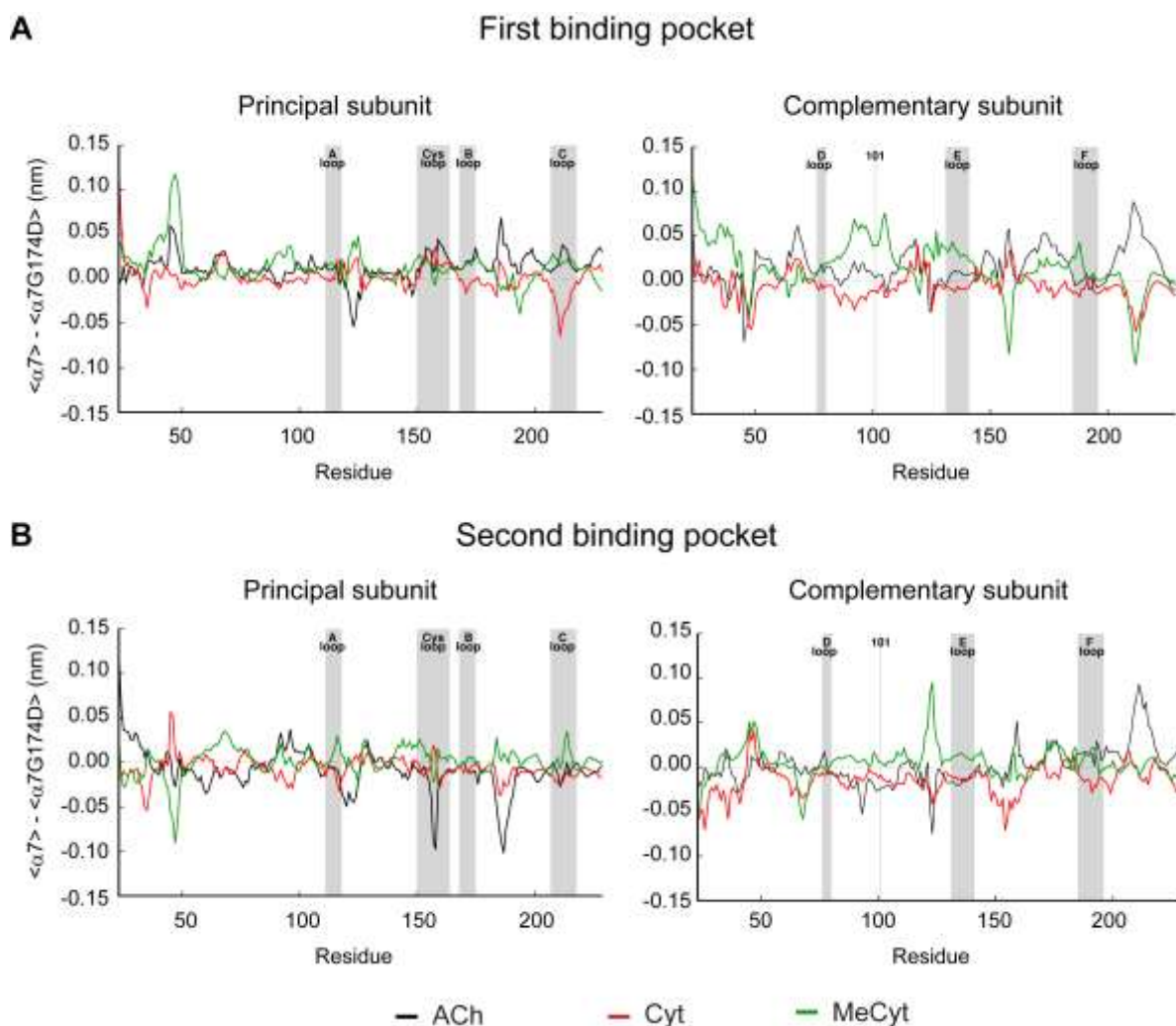
**Figure S7.** Sequence alignment for loop B and  $\beta$ 3-strand residues of nAChR subunits. The arginine residue in  $\beta$ 3-strand is highly conserved in the nAChR family, whereas the aspartate in loop B is conserved by subunits that contribute the principal side of the agonist sites in heteromeric receptors, except for the  $\alpha$ 9 and  $\alpha$ 10 subunits.

	Loop B		$\beta$ -strand 3
nAChR $\alpha$ 1 subunit	K L G T W T Y <b>D</b> G S V V A I N P E S D	nAChR $\alpha$ 1 subunit	G V K K I <b>H</b> I P S D K
nAChR $\alpha$ 2 subunit	K F G S W T Y <b>D</b> K A K I D L E Q M E Q	nAChR $\alpha$ 2 subunit	N I T S L R V P S E M
nAChR $\alpha$ 3 subunit	K F G S W S Y <b>D</b> K A K I D L V L I G S	nAChR $\alpha$ 3 subunit	G A E F M R V P A Q K
nAChR $\alpha$ 4 subunit	K F R S W T Y <b>D</b> H T E I D M V L M T P	nAChR $\alpha$ 4 subunit	G V N I L R I P A K R
nAChR $\alpha$ 5 subunit	K F G S W T Y <b>D</b> G S Q V D I I L E D Q	nAChR $\alpha$ 5 subunit	G I K V I R V P S D S
nAChR $\alpha$ 6 subunit	K F G S W T Y <b>D</b> K A E I D L L I I G S	nAChR $\alpha$ 6 subunit	G I E T L R V P A D K
nAChR $\alpha$ 7 subunit	K F G S W S Y <b>G</b> G W S L D L Q M Q E	nAChR $\alpha$ 7 subunit	G V K T V R F P D G Q
nAChR $\alpha$ 9 subunit	T F G S W T Y <b>N</b> G N Q V D I F N A L D	nAChR $\alpha$ 9 subunit	G L D S I R I P S D L
nAChR $\alpha$ 10 subunit	T F G S W T H <b>G</b> G H Q L D V R P R G A	nAChR $\alpha$ 10 subunit	G L D A I R I P S S L
nAChR $\beta$ 2 subunit	K F R S W T Y <b>D</b> R T E I D L V L K S E	nAChR $\beta$ 2 subunit	N M K K V R L P S K H
nAChR $\beta$ 4 subunit	K F G S W T Y <b>D</b> K A K I D L V N M H S	nAChR $\beta$ 4 subunit	N V T S I R I P S E L
nAChR $\epsilon$ subunit	I F R S Q T Y <b>N</b> A E E V E F T F A V D	nAChR $\epsilon$ subunit	G I E T L R V P S E L
nAChR $\delta$ subunit	K F S S L K Y <b>T</b> A K E I T L S L K Q D	nAChR $\delta$ subunit	N I S V L R L P P D M
nAChR $\gamma$ subunit	I F G S Q T Y <b>S</b> T N E I D L Q L S Q E	nAChR $\gamma$ subunit	G L W V L <b>R</b> V P S T

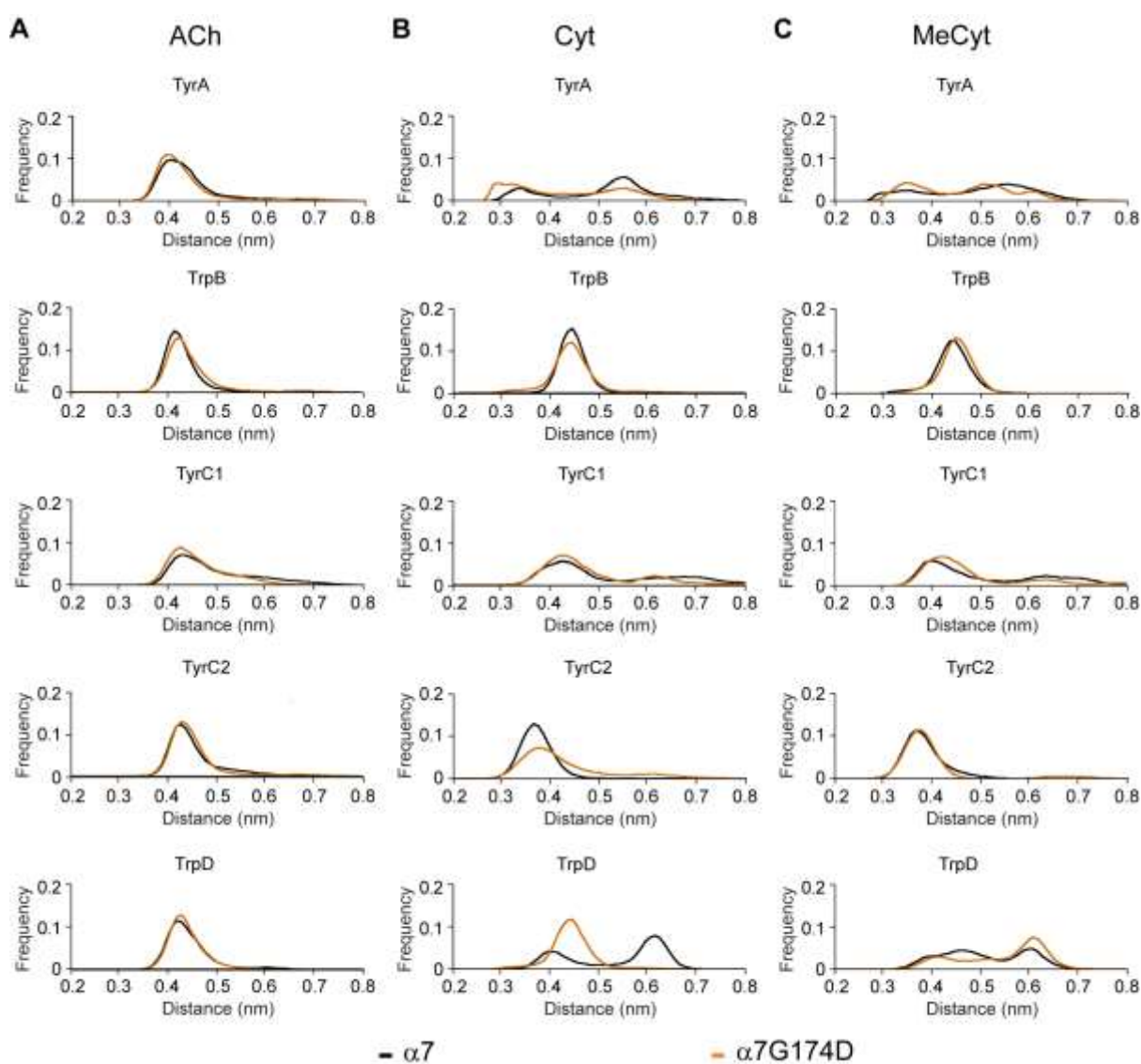
**Figure S8.** Statistical correlations for the residue in position 174 for the  $\alpha 7$  and mutant  $\alpha 7G174D$  receptor complexes with ACh, cytosine or 10-methylcytosine bound. Correlated motions for the residue in position 174 in the **A)** first and **B)** second binding pocket. The correlations between the C $\alpha$  atom of residue 174 in the principal subunit and all the remaining C $\alpha$  atoms are shown. Note that the atoms that systematically move along the opposite direction as residue 174 have a correlation value of -1 whereas those systematically moving along the same direction show a correlation of 1. The atoms whose movements relative to residue 174 are uncorrelated present a correlation value of 0. The location of residue 174 is highlighted with a sphere. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



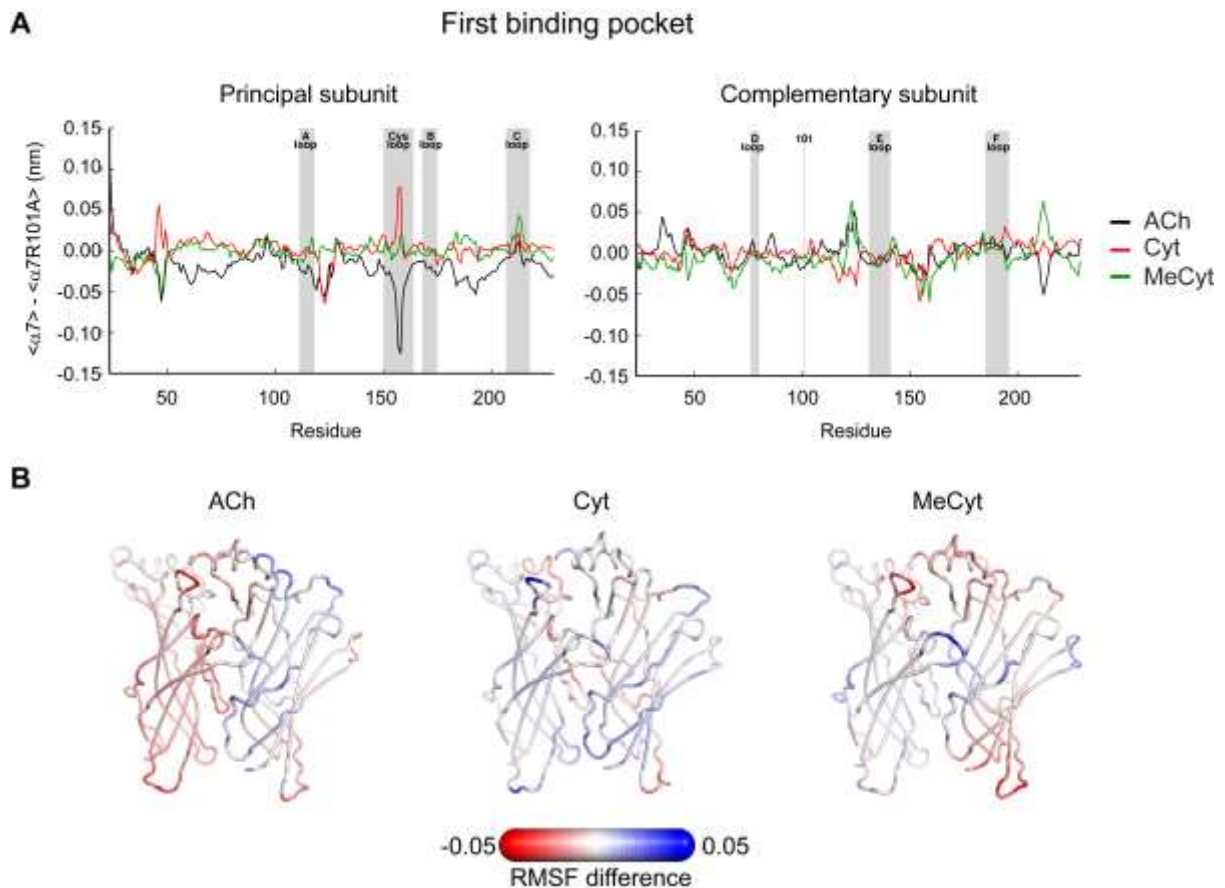
**Figure S9.** Average change in C $\alpha$  RMSF between the  $\alpha 7$  and  $\alpha 7G174D$  systems with ACh, cytosine or 10-methylcytosine bound in the **A)** first and **B)** second binding pocket. The RMSF differences for the principal (left side image) and complementary (right side image) subunits are shown for the two agonist binding sites. Note that the complexes with cytosine and, particularly 10-methylcytosine, the largest dynamical differences in the binding pockets were located around the R101 region and in loop E. Please zoom into the image for detailed visualization. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



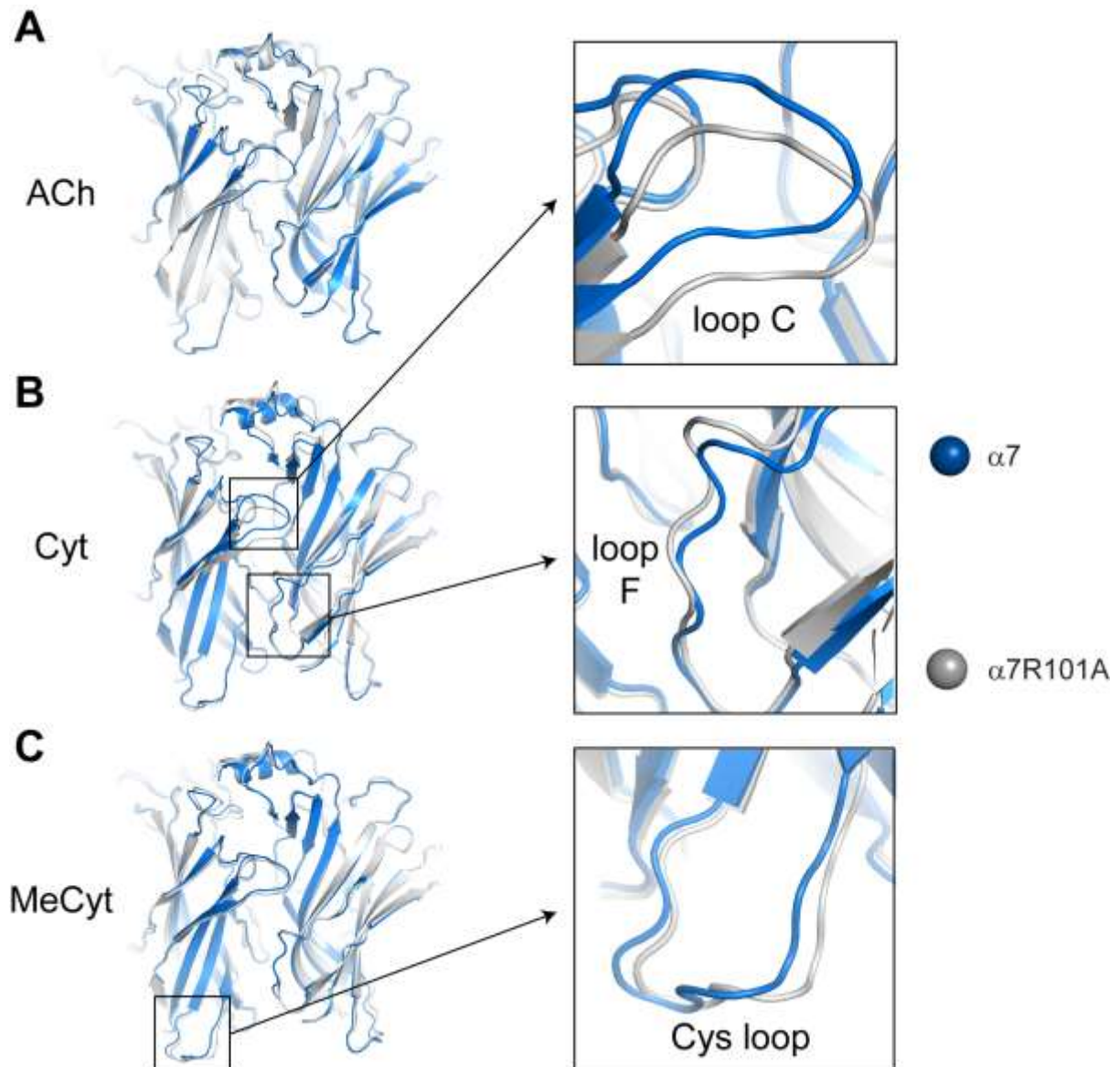
**Figure S10.** Distribution of the distance between **A)** ACh, **B)** cytosine and **C)** 10-methylcytosine and the main conserved aromatic residues lining the binding pockets in the  $\alpha 7$  (black line) and  $\alpha 7G174D$  (orange line) systems. Overall distribution of the distance between the sidechain of TyrA ( $\alpha 7115$ ), TrpB ( $\alpha 7W171$ ), TyrC1 ( $\alpha 7Y210$ ), TyrC2 ( $\alpha 7Y217$ ) or TrpD ( $\alpha 7W77$ ) and the charged N atom of the agonists. The histograms reflect the distances over the two binding pockets. Note that for ACh-bound systems, the differences in the Y115 and W77 distance profiles between the  $\alpha 7$  and  $\alpha 7G174D$  receptors are subtle, probably because the high mobility and small size of ACh makes the binding interactions of this agonist more tolerant of structural changes. In contrast, for cytosine and 10-methylcytosine, differences in the distance profiles are observed. In particular, for cytosine, the changes in the Y115 and W77 profiles between the  $\alpha 7$  and  $\alpha 7G174D$  receptors are substantial. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



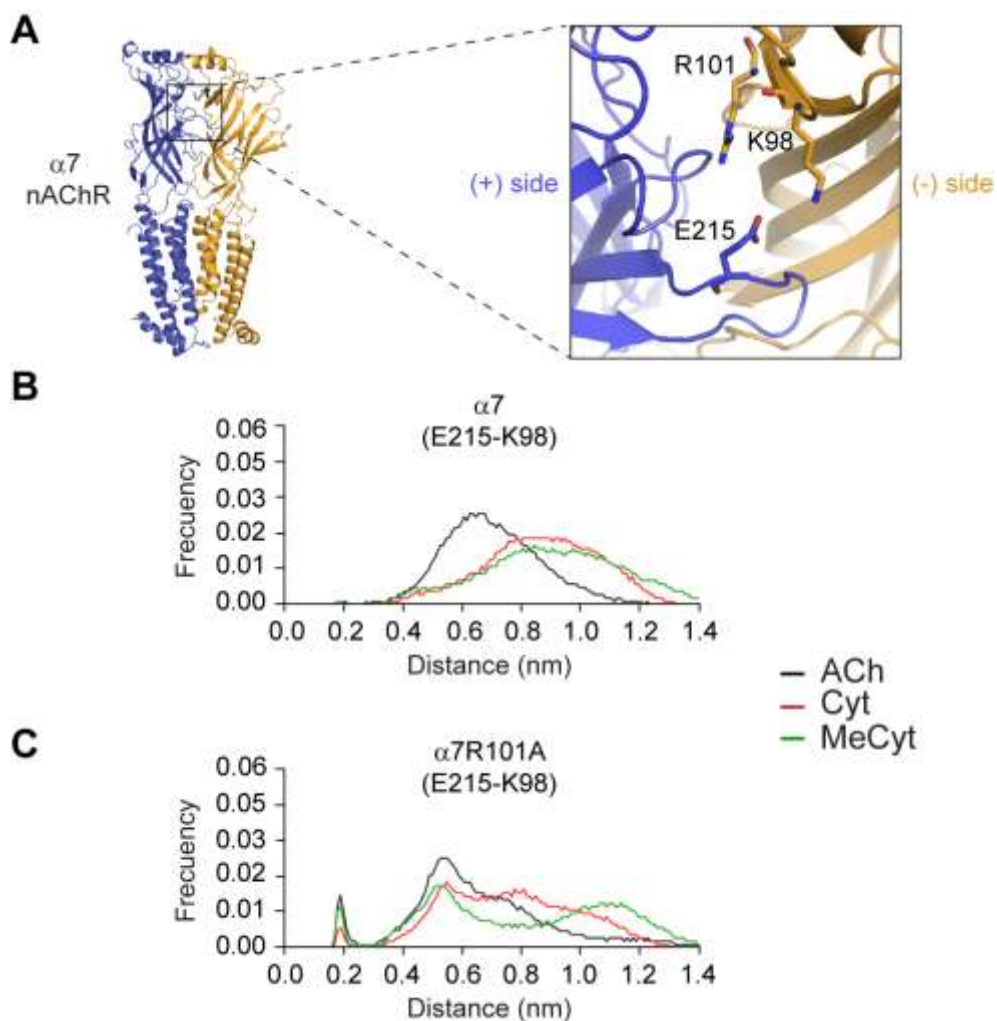
**Figure S11. A)** Average change in C $\alpha$  RMSF between the  $\alpha 7$  and  $\alpha 7R101A$  systems with ACh (black line) cytosine (red line) or 10-methylcytosine (green line) bound to the second non-consecutive binding pocket of the receptors. The RMSF differences for the principal (left side image) and complementary (right side image) subunits are shown. Please zoom into the image for detailed visualization. **B)** Average RMSF difference between  $\alpha 7$  and  $\alpha 7R101A$  systems bound to ACh, cytosine or 10-methylcytosine mapped into the average structure of each system. The structure colours are related to the average C $\alpha$  RMSF: the red and blue regions correspond to the residues with the largest differences between the two systems. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.



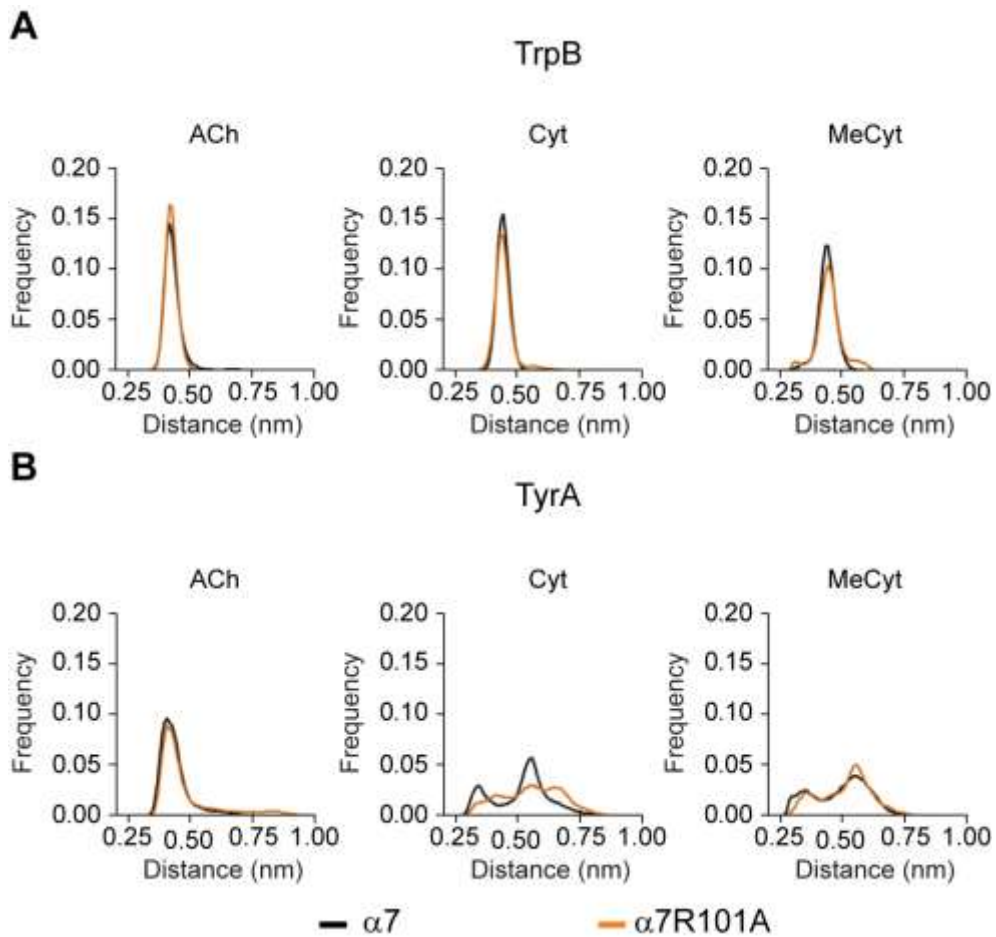
**Figure S12.** Average structure of agonist-bound  $\alpha 7$  and  $\alpha 7R101A$  complexes. The agonist simulated were **A)** ACh, **B)** cytosine and **C)** 10-methylcytosine bound to the second non-consecutive binding pocket of the receptor. In this image,  $\alpha 7R101A$  structures are colored in blue, whereas the  $\alpha 7$  ones are shown in grey. Note that for the cytosine-bound  $\alpha 7R101A$  complex, there is a clear change in the extent of loop C capping, compared to  $\alpha 7$  wild type nAChR. The structures shown here represent the average structure calculated over all replicates for each system, as described in the the Methods section of main manuscript. Abbreviations: cytosine, Cyt and 10-methylcytosine, MeCyt.



**Figure S13.** Comparison of loop C E215 and  $\beta$ 3-strand K98 interaction in the  $\alpha 7$  and  $\alpha 7R101A$  receptors. **A)** Homology model of the  $\alpha 7$  nAChR (left panel) with the inset showing a close-up view of the agonist pocket. Note position of the side chains of R101, E215 and K98. The principal and complementary subunits are coloured in blue and orange, respectively. The residues numbering refers to the UniProt sequence codes: P36544 ( $\alpha 7$  subunit cDNA). **B)** Overall distribution of the minimum distance between the sidechains of E215 and K98 in the wild type  $\alpha 7$  receptor in the presence of ACh (black line), cytosine (red line) or 10-methylcytosine (green line). **C)** Overall distribution of the minimum distance between the sidechains of E215 and K98 in the  $\alpha 7R101A$  receptor in the presence of ACh (black line), cytosine (red line) or 10-methylcytosine (green line). The histograms shown in B) and C) reflect the distances over the two binding pockets. Abbreviations: cytosine, Cyt; 10-methylcytosine, MeCyt.

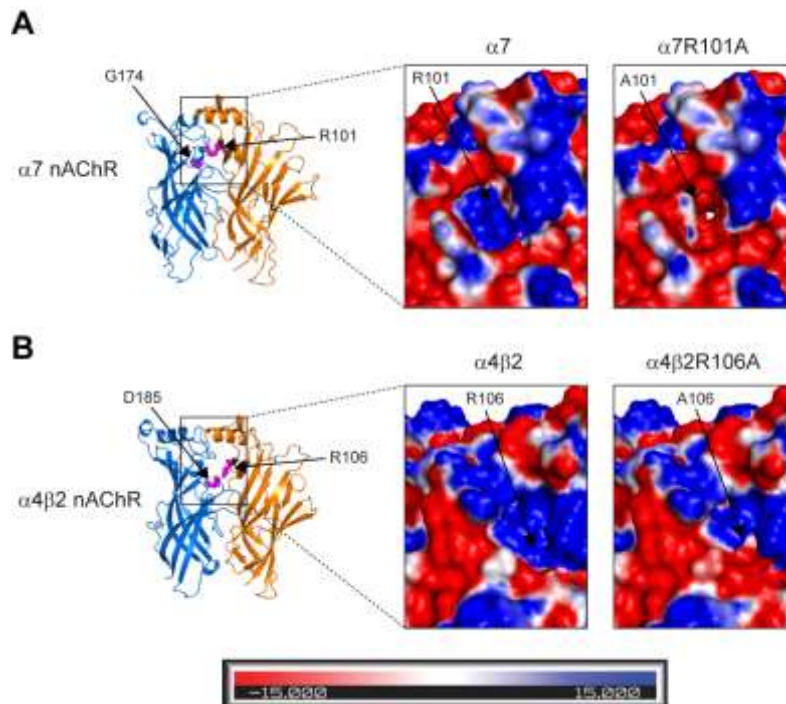


**Figure S14.** Distribution of the distance between the agonists and the main aromatic residues forming the binding pockets in the  $\alpha 7$  and  $\alpha 7R101A$  receptors. **A)** Overall distribution of the distance between the sidechain of TrpB (W171 in the  $\alpha 7$  subunit) and the charged N atom of the ligands. **B)** Overall distribution of the distance between the sidechain of TyrA (Y115 in the  $\alpha 7$  subunit) and the charged N atom of the ligands. The histograms reflect the distances over the two binding pockets. Abbreviations: cytisine, Cyt; 10-methylcytisine, MeCyt.





**Figure S15.** Electrostatic maps for  $\alpha 7$  and  $\alpha 4\beta 2$  receptors and their respective arginine mutants. **A)**  $\alpha 7$  (left panel) and  $\alpha 7R101A$  (right panel) nAChRs and **B)**  $\alpha 4\beta 2$  (left panel) and  $\alpha 4\beta 2R106A$  (right panel) receptors. Zoom of the electrostatic potential distribution on the top of the agonist site. The potential varies between  $-15$  and  $+15$   $KT/e$ , as shown in the colour-reference bar, with red and blue representing negative and positive potentials, respectively. In both  $\alpha 7$  and  $\alpha 4\beta 2$  subtypes, the substitution of the long positively charged side chain of arginine in  $\beta 3$ -strand by a short neutral side chain of alanine leads not only to a change in the shape and size of the binding site but also in the charge distribution surrounding the orthosteric binding pocket. Note, however that  $\beta 3$ -strand arginine shows distinct dynamic behaviours between the  $\alpha 7$  and  $\alpha 4\beta 2$  nAChRs (Figure S5). In the  $\alpha 4\beta 2$  nAChR, the side-chain of R106 shows reduced dynamics as it involved in a strong inter-subunit salt-bridge with D185. In the  $\alpha 7$  nAChR, the side-chain of R101 exhibits high mobility being even able to orient towards the inside of the binding pocket, which may further impact the electrostatic landscape of that region.



## 2. Supporting Tables

Table S1. Sensitivity of wild type and mutant  $\alpha 7$  nAChRs to activation by agonists. Recently, we have shown that even though cytosine and its C(10)-methyl variant share the same core-binding interactions with conserved residues in the  $\alpha 7$  and  $\alpha 4\beta 2$  nAChRs (Rego-Campello et al., 2018), 10-methylcytosine display negligible binding to  $\alpha 7$  receptors. By superimposing structural models of the agonist pocket of  $\alpha 4\beta 2$  and  $\alpha 7$  receptors bound to 10-methylcytosine, we observed differences in a layer of non-agonist binding residues in the binding pocket, namely  $\beta 2F144$  vs  $\alpha 7Q139$ ,  $\beta 2V136$  vs  $\alpha 7L131$ ,  $\beta 2A65$  vs  $\alpha 7L60$ ,  $\alpha 4T183$  vs  $\alpha 7S172$  and  $\beta 2S133$  vs  $\alpha 7T128$  (A) (Rego-Campello et al., 2018) Note that the sequence numbering that we use for  $\alpha 7$  (UniProt code P36544),  $\alpha 4$  (UniProt code P43681) and  $\beta 2$  (UniProt code P17787) cDNAs includes the signal sequence. Given that these residues could modify hydrogen bonding patterns, hydrophobicity or volume of the agonist pocket, which could affect agonist binding, we tested if making  $\alpha 7$  receptors  $\alpha 4\beta 2$ -like in respect of these residues could increase the potency of 10-methylcytosine for  $\alpha 7$  receptors. However, functional assays of  $\alpha 7$  nAChR bearing these  $\alpha 4\beta 2$  residues do not display higher sensitivity to activation 10-methylcytosine. Whole-cell current responses to agonist were measured using *Xenopus laevis* oocyte two electrode voltage clamping electrophysiology. The agonists tested were ACh, cytosine and 10-methylcytosine. The nucleus of oocytes was injected with wild type or mutant  $\alpha 7$  subunit cDNA. Data were fitted with the empirical Hill equation, as described in Methods section of the main manuscript. The estimated parameters  $EC_{50}$  and  $I_{max}/I_{AChMax}$  represent the means (95% CI) of 8-10 independent experiments carried out using 5 different oocyte donors. Statistical analysis was performed by comparing the estimated values of WT and mutant  $EC_{50}$  and  $I_{max}/I_{AChMax}$  using ANOVA followed by Dunnett's post-test. Asterisks indicate level of statistical difference from wild type (\* $p < 0.05$ ). ND, not determined.

Receptor	ACh		Cytosine		10-Methylcytosine	
	$EC_{50}$ ( $\mu M$ ) (95% CI)	$I_{max}/I_{AChMax}$ (95% CI)	$EC_{50}$ ( $\mu M$ ) (95% CI)	$I_{max}/I_{AChMax}$ (95% CI)	$EC_{50}$ ( $\mu M$ ) (95% CI)	$I_{max}/I_{AChMax}$ (95% CI)
$\alpha 7$	82.4 (71-95)	1	29.64 (24-37)	0.93 (0.9-0.94)	643 (493-837)	0.55 (0.4-0.7)
$\alpha 7L60A$ , $L131V$	50.0 (19-134)	1	66.2 (23-191)	0.9 (0.7-1.2)	ND	0.70
$\alpha 7Q139F$	462* (340-628)	1	137* (21-254)	1.11 (1.0-1.2)	504 (301-545)	0.35 (0.1-0.6)
$\alpha 7T128S$ , $S172T$	367.3* (232-581)	1	91.2* (57-271)	1.10 (0.8-1.4)	972.7* (321-2944)	0.74 (0.5-1.0)

**Table S2.** An inter-subunit salt-bridge between  $\alpha 4$ D185 and  $\beta 2$ R106 is necessary for receptor expression. Dismantling the  $\alpha 4$ D185- $\beta 2$ R106 salt-bridge by the introduction of D185G or R106A ablated functional expression of the  $(\alpha 4)_2(\beta 2)_3$  and decreased functional expression of  $(\alpha 4)_3(\beta 2)_2$  by about 14-fold. To estimate functional expression, we compared the amplitude of the currents elicited by 1mM ACh (a maximal ACh concentration at  $(\alpha 4)_3(\beta 2)_2$  and  $(\alpha 4)_2(\beta 2)_3$  receptors as well as at mutant  $(\alpha 4^{D185G})_2(\beta 2)_3$  and  $(\alpha 4)_2(\beta 2^{R106A})_3$  receptors). The overall amount of cDNA mixture injected was kept the same for all receptors assayed (5 ng). To favour the expression of the  $3\alpha 4$ - $2\beta 2$  stoichiometry, the cDNAs were injected at 10 $\alpha 4$ : 1 $\beta 2$  ratio, whereas for the  $2\alpha 4$ - $3\beta 2$  stoichiometry, the ratio was 1  $\alpha 4$  ; 10  $\beta 2$ . Maximal currents were elicited with 1 mM ACh, a maximal ACh concentration at wild type and the mutant  $\alpha 4\beta 2$  receptors. The current responses of mutant and wild type receptors were measured on the same day using the automated two-electrode voltage clamp HiClamp system, as described in the Methods section of the manuscript. The I/IACHmax data shown are the means  $\pm$  SEM obtained from 18 recordings carried out using 6 different oocyte donors (n = 18; N = 6). The concentration-response data for ACh were fitted with the Hill equation as detailed in the Method section of the main manuscript; the EC<sub>50</sub> shown were obtained from n = 10 independent recordings and using N = 5 oocyte donors. The reduced level of functional expression impeded the construction of reliable activation concentration-response relationships for cytisine and 10-methylcytisine. These ligands behave as poorly efficacious partial agonist at the two forms of the  $\alpha 4\beta 2$  receptors. Therefore, to assess the impact of dismantling the D-R salt bridge on the functional potency of cytisine and 10-methylcytisine, we evaluated the ability of these ligands to act as competitive antagonists. This strategy is based on the rationale that partial agonists, such as cytisine and 10-methylcytisine, fully occupy the agonist binding site while having low efficacy in activating the receptor. When in the binding site, a ligand will prevent other agonists from binding and activating the receptor, thus in this circumstance the partial agonist also acts as a partial competitive antagonist. Neither D185G nor R106A affected the inhibitory potency of cytisine or 10-methylcytisine, compared to wild type (n = 8; N= 5). [<sup>3</sup>H]Epibatidine binding assays on cells expressing  $\alpha 4\beta 2$  receptors showed a five-fold decrease in Bmax but no changes in Kd. Data shown represent the mean of three independent experiments carried out in triplicate and using three different batches of cultured cell (n = 3; N = 3). Collectively, the data indicates that abolishing the D185-R106 salt bridge disturbs cell surface expression but not the binding affinity or efficacy of cytisine or 10-methylcytisine.

<b>Whole-cell recordings</b>				
<b>Receptor</b>	<b>I<sub>AChMax</sub> ±SEM μA</b>	<b>ACh EC<sub>50</sub> (μM) (95% CI)</b>	<b>Cytisine IC<sub>50</sub> (μM) (95% CI)</b>	<b>10-methylcytisine IC<sub>50</sub> (μM) (95% CI)</b>
(α4) <sub>3</sub> (β2) <sub>2</sub>	1.8±0.6	60 (48-69)	13 (9-19)	13 (6-28)
(α4 <sup>D185G</sup> ) <sub>3</sub> (β2) <sub>2</sub>	0.11±0.06	67 (54-84)	21 (11-40)	14 (7-29)
(α4) <sub>3</sub> (β2 <sup>R106A</sup> ) <sub>2</sub>	0.09±0.01	53 (26-47)	25 (10-61)	14 (11-18)
(α4) <sub>2</sub> (β2) <sub>3</sub>	0.8±0.1	7 (5.2-8.2)	0.56 (0.3-1.0)	0.73 (0.3-2.1)
(α4 <sup>D185G</sup> ) <sub>2</sub> (β2) <sub>3</sub>	0.05±0.02	ND	ND	ND
(α4) <sub>2</sub> (β2 <sup>R106A</sup> ) <sub>3</sub>	0.05±0.009	ND	ND	ND
<b>[<sup>3</sup>H]Epibatidine Binding</b>				
<b>Receptor</b>	<b>K<sub>d</sub> ± SEM (nM)</b>		<b>B<sub>max</sub> ± SEM (fmol/mg of prot.)</b>	
α4β2	0.106 ±0.012		1219 ± 68	
α4D185Gβ2	0.149±0.013		269 ± 107	

**Table S3.** Microscopic currents of wild type and mutant  $\alpha 7$  nAChR.

<b>Agonist</b>	<b>Receptor</b>	$\tau_{\text{open}}$ (ms)	$\tau_{\text{burst}}$ (ms)	<b>n</b>	<b>N</b>
ACh	$\alpha 7$	$0.27 \pm 0.02$	$0.33 \pm 0.06$	6	5
	$\alpha 7\text{G174D}$	$0.26 \pm 0.10$	$0.69^* \pm 0.17$	5	5
	$\alpha 7\text{R101A}$	$0.29 \pm 0.04$	$1.41^* \pm 0.41$	5	5
Cytisine	$\alpha 7$	$0.28 \pm 0.09$	$0.72 \pm 0.12$	6	5
	$\alpha 7\text{G174D}$	$0.31 \pm 0.08$	$1.25^* \pm 0.30$	4	5
	$\alpha 7\text{R101A}$	$0.29 \pm 0.10$	$2.00^* \pm 0.26$	7	5

$\tau_{\text{open}}$  and  $\tau_{\text{burst}}$  correspond to the longest components of the corresponding open time and burst duration histograms. Values are mean  $\pm$  SD. n: number of independent experiments, each from different cell patches. N: number of cell transfections. Statistical significance was determined by comparing the mean value in the mutant receptor respect to the mean value in the wild type  $\alpha 7$  receptor for each agonist, by two-tailed Student's t-test; \*, denotes statistical difference at  $p < 0.05$ . N corresponds to number of transfected cell batches used, and n is the number of recordings carried out.