## Supporting Information

Molecular determinants of α-conotoxin potency for inhibition of human and rat α6β4 nicotinic acetylcholine receptors

## Arik J. Hone<sup>1</sup>, Todd T. Talley<sup>2</sup>, Janet Bobango<sup>3</sup>, Cesar Huidobro Melo<sup>1</sup>, Fuaad Hararah<sup>1</sup>, Joanna Gajewiak<sup>1</sup>, Sean Christensen<sup>1</sup>, Peta J. Harvey<sup>4</sup>, David J. Craik<sup>4</sup>, J. Michael McIntosh<sup>1,5,6</sup>

## Running title: Determinants of α-Ctx potency for human α6β4 nAChRs

<sup>1</sup>Departments of Biology and <sup>5</sup>Psychiatry, University of Utah, Salt Lake City, Utah, USA; <sup>6</sup>George E. Whalen Veterans Affairs Medical Center, Salt Lake City, Utah, USA; <sup>2</sup>T3 Biosciences, Moses Lake, Washington, USA; <sup>3</sup>Department of Biomedical and Pharmaceutical Sciences, The University of Montana, Missoula, Montana, USA; <sup>4</sup>University of Queensland, Institute for Molecular Bioscience, Brisbane, Queensland, Australia.

Table S1	
Crystallography statistics	
Data collection	
Resolution range (Å)	48.82 - 2.34 (2.42 - 2.34)
Space group	$C 2 2 2_1$
Unit cell	145.12 147.89 146.31 90 90 90
Completeness (%)	98.9
Unique reflections	66,040 (6,263)
Wilson B-factor ( $Å^2$ )	34.8
Refinement	
Number of residues	
AChBP	1032
PeIA	68
Water	332
<i>R</i> (work) (%)	0.1959
<i>R</i> (free) (%)	0.228
RMS bonds (Å)	0.008
RMS angles	1.29
Ramachandran favored (%)	97.1
Ramachandran allowed (%)	2.9
Ramachandran outliers (%)	0.0
Average $B$ (Å <sup>2</sup> )	39.89
AChBP	39.92
PeIA	63.31
Water	38.66

Statistical analysis of [PI3A]PeIA structures	
Experimental restraints	
total no. distance restraints	90
intraresidue	36
sequential	36
medium range, <i>i-j</i> <5	14
long range, <i>i-j</i> ≥5	4
hydrogen bond restraints	4
dihedral angle restraints	
phi	8
psi	6
Violations	
NOE violations exceeding 0.2 Å	0
Dihedral violations exceeding 2.0 Å	0
Rms deviation from mean structure, Å	
backbone atoms	$0.60\pm0.16$
all heavy atoms	$1.13\pm0.22$
Stereochemical quality <sup>b</sup>	
Residues in most favored Ramachandran region, %	$93.2\pm5.8$
Ramachandran outliers, %	$0.0 \pm 0.0$
Unfavorable sidechain rotamers, %	$17.7 \pm 3.5$
Clash score, all atoms	$0.5 \pm 1.5$
Overall MolProbity score	$1.8 \pm 0.2$

Statistical analysis of [P13A]PeIA structures

<b>A</b> Human Alpha6 Rat Alpha6	- E E R L F H - E E Q L F H	10 I K L F S H Y N I T L F A H Y N	20 Q F I R P V E N V R F I R P V E N V	30 7 S D P V T V H F E 7 S D P V T V H F E	40 V A I T Q L A N V D L A I T Q L A N V D	50 E V N Q I M E T E V N Q I M E T
Human Alpha6 Rat Alpha6	5 NLWLRH NLWLRHV	60 WNDYKLR WKDYRLC	TO TO TO TO TO TO TO TO TO TO TO TO TO T	80 I E T L R V P A D K I E T L R V P A D N	90 I W K P D I V L Y N I W K P D I V L Y N	100 N A V G D F Q V N A V G D F Q V
Human Alpha6 Rat Alpha6	5 EGKTKAL EGKTKAL	110 . L K Y N G M I . L K Y D G V I	120 TWTPPAIFI TWTPPAIFI	130 X S S C P M D I T F X S S C P M D I T F	140 F P F D H Q N C S L F P F D H Q N C S L	150 K F G S W T Y D K F G S W T Y D
Human Alpha6 Rat Alpha6	* 6 KAEIDLL KAEIDLL	160 2 I I G S K V E 2 I I G S K V E	170 0 M N D F W E N S 1 0 M N D F W E N S 1	180 EWEIDASGY EWEIVDASGY	* 190 K H D I K Y N C C E K H D I K Y N C C E	* 200   E I Y T D I T Y   E I Y T D I T Y
Human Alpha6 Rat Alpha6	5 SFYIRRL SFYIRRL	210 2 PMFYTIN 2 PMFYTIN	220    L I I P C L F I S    L I I P C L F I S	230 8 F L T V L V F Y L 8 F L T V L V F Y L	240 P S D C G E K V T L P S D C G E K V T L	250 C I S V L L S L C I S V L L S L
Human Alpha6 Rat Alpha6	TVFLLVI TVFLLVI	260 TETIPST TETIPST	270 S L V V P L V G I S L V I P L V G I	280 E Y L L F T M I F V E Y L L F T M I F V	290 T L S I V V T V F V T L S I V V T V F V	300 L N I H Y R T P L N I H Y R T P
В		10	20	30	40	50
Human beta4 Rat beta4	A N A E E K L A N A E E K L	M D D L L N K M D D L L N K	T R Y N N L I R P T R Y N N L I R P	A T S S S Q L I S A T S S S Q L I S	I K L Q L S L A Q L I R L E L S L S Q L	I SVNEREQ ISVNEREQ
Human beta4 Rat beta4	IMTTNVW IMTTSIW	*60 L K Q E W T D L K Q E W T D *	70 Y R L Y R L A W N S S C	80 Y E G V N I L R I Y E G V N I L R I	90 PAKRIWLPDI PAKRVWLPDI	100 V L Y N N A D G V L Y N N A D G
Human beta4 Rat beta4	T Y E V S V Y T Y E V S V Y	110 * * T N L I V R S T N V I V R S *	120 N G S V L W L P P N G S I Q W L P P	130 A I Y K S A C K I A I Y K S A C K I	140 E V K Y F P F D Q Q E V K H F P F D Q Q	150 N C T L K F R S N C T L K F R S
Human beta4 Rat beta4	WTYDHTE WTYDHTE	160 I D M V L M T I D M V L K S	170 PTASMDDFT PTAIMDDFT	Image: 180   P S G E W D I V A   P S G E W D I V A	190 L P G R R T V N P Q L P G R R T V N P Q	200 D P S Y V D V T D P S Y V D V T
Human beta4 Rat beta4	Y D F I I K R Y D F I I K R	210 K P L F Y T I K P L F Y T I	220 N L I I P C V L T N L I I P C V L I	230 T L L A I L V F Y T S L A I L V F Y	240 L P S D C G E K M T L P S D C G E K M T	250 L C I S V L L A L C I S V L L A

FIGURE S1. Sequence homology of human and rat  $\alpha 6$  and  $\beta 4$  subunits. (A) Sequence alignment of human and rat  $\alpha 6$  subunits. A pairwise analysis of the extracellular ligand-binding domains (residues 1-206) indicated 192 (93%) conserved identities. Red asterisks indicate residues that have previously been shown in functional assays to be important for  $\alpha$ -Ctx binding to rat  $\alpha 6/\alpha 3\beta 2\beta 3$  nAChRs (1). (B) Sequence alignment of human and rat  $\beta 4$  subunits. A pairwise analysis of the extracellular ligand-binding domains (residues 1-208) indicated 193 (93%) conserved identities. Red asterisks indicate residues that have previously been shown in functional assays to be important for  $\alpha$ -Ctx binding to  $\alpha 3\beta 4$  nAChRs (2). Asterisks above the sequence line indicate residues identified in human receptors and those below indicate rat receptors. Alignments and sequence comparisons were performed using MacVector.

		10		20	30	40	50
Human beta2	TDTE	ERLVEH	LLDPSRY	NKLIRPA	ATNGSELVTV	QLMVSLAQLIS	SVHEREQ
Rat beta2	TDTE	ERLVEH	LLDPSRY	NKLIRPA	ATNGSELVTV	<b>QLMVSLAQLIS</b>	SVHEREQ
		*60		70	80	90	100
Human beta2	IMTTI	N V W L T Q	EWEDYRL	TWKPEEI	FDNMKKVRLF	<b>SKHIWLPDVVI</b>	YNNADG
Rat beta2	IMTT	N V W L T Q	EWEDYRL	TWKPEDI	FDNMKKVRLF	<b>SKHIWLPDVVI</b>	LYNNADG
		*					
		200000		10.2121	5000 BT		10.000
		110	*	120	130	140	150
Human beta2	MYEV	SFYSNA	VVSYDGS	IFWLPPA	AIYKSACKIE	E V K H F P F D Q Q N C	CTMKFRS
Rat beta2	MYEV	SFYSNA	VVSYDGS	IFWLPPA	AIYKSACKIE	EVKHFPFDQQNC	CTMKFRS
			**	* *			
		1(0		170	100	100	200
Harmon hada2	WTYDI					190	
Human beta2	WIYD		V L K S E V A	SLDDFI	SGEWDIVAI	PGRENENPDDS	S I Y V D I I
Rat Deta2	WIYD	KIEIDL	VLKSDVA	SLDDFI	P S G E W D I I A I	PGRKNENPDDS	SIYVDII
		210		220	230	240	250
Human heta?	VDFI	210	FVTINLI	220	230	240	250
Human beta2 Rat beta2	YDFI	210 IRRKPL IRRKPL	FYTINLI	220 I P C V L I T	230 <b>FSLAILVFYI FSLAILVFYI</b>	240 . P S D C G E K M T L C . P S D C G E K M T L C	250 C I S V L L A C I S V L L A
Human beta2 Rat beta2	YDFI YDFI	210 I R R K P L I R R K P L	FYTINLI FYTINLI	220 I P C V L I T I P C V L I T	230 <b>F S L A I L V F Y I</b> <b>F S L A I L V F Y I</b>	240 . P S D C G E K M T L C . P S D C G E K M T L C	250 C I S V L L A C I S V L L A
Human beta2 Rat beta2	YDFI YDFI	210 I R R K P L I R R K P L	FYTINLI FYTINLI	220 I P C V L I T I P C V L I T	230 F S L A I L V F Y I F S L A I L V F Y I	240 . P S D C G E K M T L C . P S D C G E K M T L C	250 C I S V L L A C I S V L L A
Human beta2 Rat beta2	YDFI YDFI	210 I R R K P L I R R K P L 260	F Y T I N L I F Y T I N L I	220 I P C V L I T I P C V L I T 270	230	240 . P S D C G E K M T L C . P S D C G E K M T L C . 290	250 C I S V L L A C I S V L L A 300
Human beta2 Rat beta2 Human beta2	YDFI YDFI	210 I R R K P L I R R K P L 260 L L L I S K	F Y T I N L I F Y T I N L I I V P P T S L	220 I P C V L I T I P C V L I T 270 D V P L V G I	230 T S L A I L V F Y I T S L A I L V F Y I 280 X Y L M F T M V L V	240 . P S D C G E K M T L C . P S D C G E K M T L C . P S D C G E K M T L C . 290 ? T F S I V T S V C V I	250 C I S V L L A C I S V L L A 300 L N V H H R S

FIGURE S2. Sequence homology of human and rat  $\beta 2$  subunits. (A) Sequence alignment of human and rat  $\beta 2$  subunits. A pairwise analysis of the extracellular ligand-binding domains (residues 1-208) indicated 205 (99%) conserved identities. Red asterisks indicate residues that have previously been shown in functional assays to be important for  $\alpha$ -Ctx binding to human (2) and rat  $\alpha 3\beta 2$  nAChRs (3-5). Asterisks above the sequence line indicate residues identified in human receptors and those below indicate rat receptors. Alignments and sequence comparisons were performed using MacVector.



FIGURE S3. Sequence comparison of the *Aplysia californica* AChBP with nAChR  $\beta$ 4 subunits, and the effect of Met substitution of human  $\beta$ 4<sub>Leu119</sub> on the potency of PeIA. (A) Sequence alignment of the AChBP with human and rat  $\beta$ 4 subunits. Note that for key residues 110, 118, and 119 of the ligandbinding pocket, only position 119 varies significantly among the three sequences. (B) Concentrationresponse analysis of the potency of PeIA on mutant human  $\alpha 6/\alpha 3\beta 4_{L119M}$  nAChRs. PeIA inhibited  $\alpha 6/\alpha 3\beta 4_{L119M}$  nAChRs with and IC<sub>50</sub> of 38.5 (34.4-43.1) nM. Values in parentheses indicate the 95% confidence interval and the error bars represent  $\pm$  SDM of the data obtained from 4 individual oocytes. (C) Current traces showing the inhibition of the ACh-evoked responses by the indicated concentrations of PeIA. The traces have been concatenated for brevity. C indicates a control response in the absence of PeIA. Alignments and sequence comparisons were performed using MacVector; numbering follows that of the AChBP sequence.



FIGURE S4. Concentration-response relationship for activation of human  $\alpha 6/\alpha 3\beta 4$  and  $\alpha 6/\alpha 3\beta 4_{L119Q}$  nAChRs by acetylcholine. *Xenopus laevis* oocytes expressing  $\alpha 6/\alpha 3\beta 4$  or  $\alpha 6/\alpha 3\beta 4_{L119Q}$  nAChRs were subjected to TEVC electrophysiology and the EC<sub>50</sub> values determined for activation by acetylcholine as described in Experimental Procedures. (A) Concentration-response analysis of ACh-gated currents obtained by applying ascending concentrations of agonist. Acetylcholine activated  $\alpha 6/\alpha 3\beta 4$  nAChRs with an EC<sub>50</sub> value of 303 (267-344)  $\mu$ M and a Hill slope of 1.3 (1.1-1.5) (n=6) and  $\alpha 6/\alpha 3\beta 4_{L119Q}$  mutant nAChRs with an EC<sub>50</sub> value of 396 (355-441)  $\mu$ M and a Hill slope of 1.7 (1.4-2.0) (n=7). The values in parentheses denote the 95% confidence interval. Error bars denote  $\pm$  SDM. (B) Representative current traces for activation  $\alpha 6/\alpha 3\beta 4$  nAChRs by 300 nM through 10 mM ACh. (C) Representative current traces for activation of  $\alpha 6/\alpha 3\beta 4_{L119Q}$  nAChRs by 300 nM through 10 mM acetylcholine. ACh was applied every 65 sec from the lowest to highest concentration and each current trace is 30 sec in duration.

## Reference

- 1. McIntosh, J. M., Azam, L., Staheli, S., Dowell, C., Lindstrom, J. M., Kuryatov, A., Garrett, J. E., Marks, M. J., and Whiteaker, P. (2004) Analogs of alpha-conotoxin MII are selective for alpha6-containing nicotinic acetylcholine receptors. *Mol Pharmacol* **65**, 944-952
- 2. Cuny, H., Kompella, S. N., Tae, H. S., Yu, R., and Adams, D. J. (2016) Key Structural Determinants in the Agonist Binding Loops of Human beta2 and beta4 Nicotinic Acetylcholine Receptor Subunits Contribute to alpha3beta4 Subtype Selectivity of alpha-Conotoxins. *J Biol Chem* **291**, 23779-23792
- 3. Dutertre, S., Nicke, A., and Lewis, R. J. (2005) Beta2 subunit contribution to 4/7 alphaconotoxin binding to the nicotinic acetylcholine receptor. *J Biol Chem* **280**, 30460-30468
- 4. Shiembob, D. L., Roberts, R. L., Luetje, C. W., and McIntosh, J. M. (2006) Determinants of alpha-conotoxin BuIA selectivity on the nicotinic acetylcholine receptor beta subunit. *Biochemistry* **45**, 11200-11207
- 5. Harvey, S. C., McIntosh, J. M., Cartier, G. E., Maddox, F. N., and Luetje, C. W. (1997) Determinants of specificity for alpha-conotoxin MII on alpha3beta2 neuronal nicotinic receptors. *Mol Pharmacol* **51**, 336-342