

# A signal peptide missense mutation associated with nicotine dependence alters $\alpha 2^*$ -nicotinic acetylcholine receptor function

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## ABSTRACT

A cytosine to thymidine (C → T) missense mutation in the signal peptide (SP) sequence (rs2472553) of the nicotinic acetylcholine receptor (nAChR)  $\alpha 2$  subunit produces a threonine-to-isoleucine substitution (T221) often associated with nicotine dependence (ND). We assessed effects on function of  $\alpha 2^*$ -nAChR (\*\* indicates presence of additional subunits) of this mutation, which could alter SP cleavage, RNA/protein secondary structure, and/or efficiency of transcription, translation, subunit assembly, receptor trafficking or cell surface expression. Two-electrode voltage clamp analyses indicate peak current responses to ACh or nicotine are decreased 2.8–5.8-fold for putative low sensitivity (LS; 10:1 ratio of  $\alpha$ : $\beta$  subunit cRNAs injected)  $\alpha 2\beta 2$ - or  $\alpha 2\beta 4$ -nAChR and increased for putative high sensitivity (HS; 1:10  $\alpha$ : $\beta$  subunit ratio)  $\alpha 2\beta 2$ - (5.7–15-fold) or  $\alpha 2\beta 4$ - (1.9–2.2-fold) nAChR as a result of the mutation. Agonist potencies are decreased 1.6–4-fold for putative LS or HS  $\alpha 2$ (T221) $\beta 2$ -nAChR or for either  $\alpha 2^*$ -nAChR subtype formed in the presence of equal amounts of subunit cRNA, slightly decreased for LS  $\alpha 2$ (T221) $\beta 4$ -nAChR, but increased 1.4–2.4-fold for HS  $\alpha 2$ (T221) $\beta 4$ -nAChR relative to receptors containing wild-type  $\alpha 2$  subunits. These effects suggest that the  $\alpha 2$  subunit SP mutation generally favors formation of LS receptor isoforms. We hypothesize that lower sensitivity of human  $\alpha 2^*$ -nAChR to nicotine could contribute to increased susceptibility to ND. To our knowledge this is the first report of a SP mutation having a functional effect in a member of cys-loop family of ligand-gated ion channels.

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## 1. Introduction

Nicotinic acetylcholine receptors (nAChR) are important in excitatory neurotransmission both at the neuromuscular junction and throughout the nervous system. These receptors are pentameric proteins composed of highly homologous subunits that are classified as either  $\alpha$  subunits ( $\alpha 1$ – $\alpha 10$ ) or non- $\alpha$  subunits (i.e.,  $\beta 1$ – $\beta 4$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  subunits) (Lukas et al., 1999). In humans, nAChR other than the muscle-type (embryonic  $\alpha 1\beta 1\gamma\delta$ - or adult  $\alpha 1\beta 1\gamma\epsilon$ -) are composed of different permutations of nine  $\alpha$  ( $\alpha 1$ – $\alpha 10$ ) subunits and three  $\beta$  ( $\beta 2$ – $\beta 4$ ) subunits. Each subunit has an N-terminal extracellular domain (ECD) that includes a signal peptide (SP) and contains residues and structural loops that form the ligand-binding site. The ECD has a pair of disulfide-bonded cysteines separated by

13 residues that form a cys-loop motif. This motif is essential for nAChR assembly and channel gating and is also a characteristic of subunits in GABA<sub>A</sub>, glycine and 5-HT<sub>3A</sub> receptors, which collectively constitute the cys-loop superfamily of receptors (Lester et al., 2004; Sine and Engel, 2006). nAChR  $\alpha$  subunits, but not non- $\alpha$  subunits, also possess tandem cysteine residues important for ligand binding.

Because of subunit diversity, numerous nAChR subtypes could be constructed, in theory, but not all possible combinations are actually formed.  $\alpha 7^*$ -,  $\alpha 4^*$ -,  $\alpha 6^*$ -, and  $\alpha 3^*$ -nAChR subtypes but not  $\alpha 2^*$ -nAChR (where the \* indicates known or possible presence in the complex of additional subunits other than those specified) are among those having proven physiological relevance. These receptors are of fundamental importance in human disorders such as Alzheimer's disease, Parkinson's disease, nicotine dependence (ND), mood and stress disorders (Steinlein and Bertrand, 2008).  $\alpha 2^*$ -nAChR are not as well studied and understood, partly because of restricted expression of  $\alpha 2$  nAChR subunits (Ishii et al., 2005; Son and Winzer-Serhan, 2006; Wada et al., 1989). nAChR  $\alpha 2$  subunits are found to be more abundant and widely distributed in primate than in rodent brain (Aridon et al., 2006; Gotti et al., 2006; Han

Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor(s);  $I_{max}$ , peak current response; SNP, single nucleotide polymorphism; SP, signal peptide; ND, nicotine dependence; LS, low sensitivity; HS, high sensitivity; CR, concentration-response; h, human; AA, amino acid.

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et al., 2000; Quik et al., 2000; Whiteaker et al., 2009). Nonetheless, distinct and potentially important physiological roles of nAChR  $\alpha 2$  subunits/ $\alpha 2^*$ -nAChR are emerging based on studies in rodents and primates, including humans (Borghese et al., 2003; Di Resta et al., 2010; Khiroug et al., 2004; Lotfipour et al., 2013; Nakauchi et al., 2007; Pandya and Yakel, 2011).

Single nucleotide polymorphisms and variations (SNPs and SNVs) (e.g., rs2472553, rs 2043063, rs104894063; etc.) in the human (h) nAChR  $\alpha 2$  subunit gene (NCBI Reference Sequence: NM\_000742.3, Entrez Gene ID: 1135) have been evaluated and sometimes associated with drug (including nicotine) dependence, asthma, bipolar disorder, obesity, and other conditions (Corley et al., 2008; Himes et al., 2010; Kim, 2008; Philibert et al., 2009; Shi et al., 2007). In particular, an amino acid substitution in the human nAChR  $\alpha 2$  subunit first transmembrane domain (rs104894063: I279N) is linked to a form of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Aridon et al., 2006). Moreover a cytosine (C) to thymidine (T) (i.e., C  $\rightarrow$  T) missense mutation (rs2472553) in the SP of the human nAChR  $\alpha 2$  subunit gene that leads to substitution of an isoleucine (I) residue for a threonine (T) residue at amino acid position 22 (i.e., T221) is significantly associated with nicotine dependence (ND) and assessment of it based on the Fagerström Test for Nicotine Dependence (FTND) (Philibert et al., 2009; Wessel et al., 2010). In this case the risk allele at mRNA/cDNA position 674 or nucleotide position 65 (the second nucleotide of the amino acid 22 triplet codon; ACC  $\rightarrow$  ATC) is T (thymidine), the ancestral allele is C (cytosine), and the frequency of the risk (or minor) allele varies greatly among different ethnic populations. Available information from NCBI dbSNP database ([http://www.ncbi.nlm.nih.gov/projects/SNP/snp\\_ref.cgi?rs=2472553](http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2472553)) according to 1000 Genome phase 1 population (<http://www.1000genomes.org/>) indicates that rs2472553 has a minor allele T account of 549 in a sample of 1089 individuals, with a frequency of 0.2521 for allele T and of 0.7479 for allele C, respectively. Under the assumption of Hardy–Weinberg equilibrium, we predict a frequency of 55.94%, 37.71% and 6.35% for C/C, C/T and T/T genotypes, respectively, for rs2472533. This SNP is in linkage disequilibrium (LD) with another SNP (rs891398) in the nAChR  $\alpha 2$  subunit that leads to the substitution of a threonine (T) residue for an alanine (A) residue at amino acid position 125 (i.e., A125T; MAF/MinorAlleleCount: T = 0.3701/806).

Functional consequences of either mutation (T221 or A125T) are not known but we got interested in the nAChR  $\alpha 2$  subunit SP mutation (T221) as it is associated with ND. Since amino acid affected in this mutation would be typically cleaved from mature proteins we wondered what effect such a mutation will have on the function of  $\alpha 2^*$ -nAChRs. Effects on RNA/protein secondary structure, efficiency of transcription, translation, cell surface expression of functional receptor; etc., if any, may be detected in studies involving transfected cell lines and/or neurons. However, we wanted to know whether the effects of the SP mutation could be detected in *Xenopus* oocyte expressed  $\alpha 2^*$ -nAChRs. This is because we wanted to know whether oocytes could serve as models for cellular or neuronal processes that involve endoplasmic reticulum (ER) exit, Golgi processing, trafficking and assembly; and membrane insertion of ion channels or receptors. In the event an effect of the SP mutation on the function of oocyte expressed  $\alpha 2^*$ -nAChRs is detected then this expression model could further be used to assess the effects of the mutation on putative low sensitivity (LS) and high sensitivity (HS)  $\alpha 2^*$ -nAChRs. This is possible as subunit ratios could be varied (limiting one or other subunit) to drive the expression of LS- and HS-  $\alpha 2^*$ -nAChRs which could not be easily achieved in cell or neuronal expression systems. Consequently, we assessed whether nAChR containing the SP mutants or wild type (WT)  $\alpha 2$  subunits differ

pharmacologically and functionally using two-electrode voltage clamp (TEVC) recordings. Results indicate that the SP mutation T221 modulates the function of both  $\alpha 2\beta 2$ - and  $\alpha 2\beta 4$ -nAChR. This is significant, because findings indicate that the SP mutation decreases sensitivities to nicotine and acetylcholine, likely by affecting subunit ratios in  $\alpha 2^*$ -nAChR, and quite possibly increasing susceptibility to ND. These results for the first time also demonstrate that *Xenopus* oocyte expression system can be used to assay the effects of SP mutation on a nAChR subtype.

## 2. Experimental procedures

### 2.1. Bioinformatics analyses

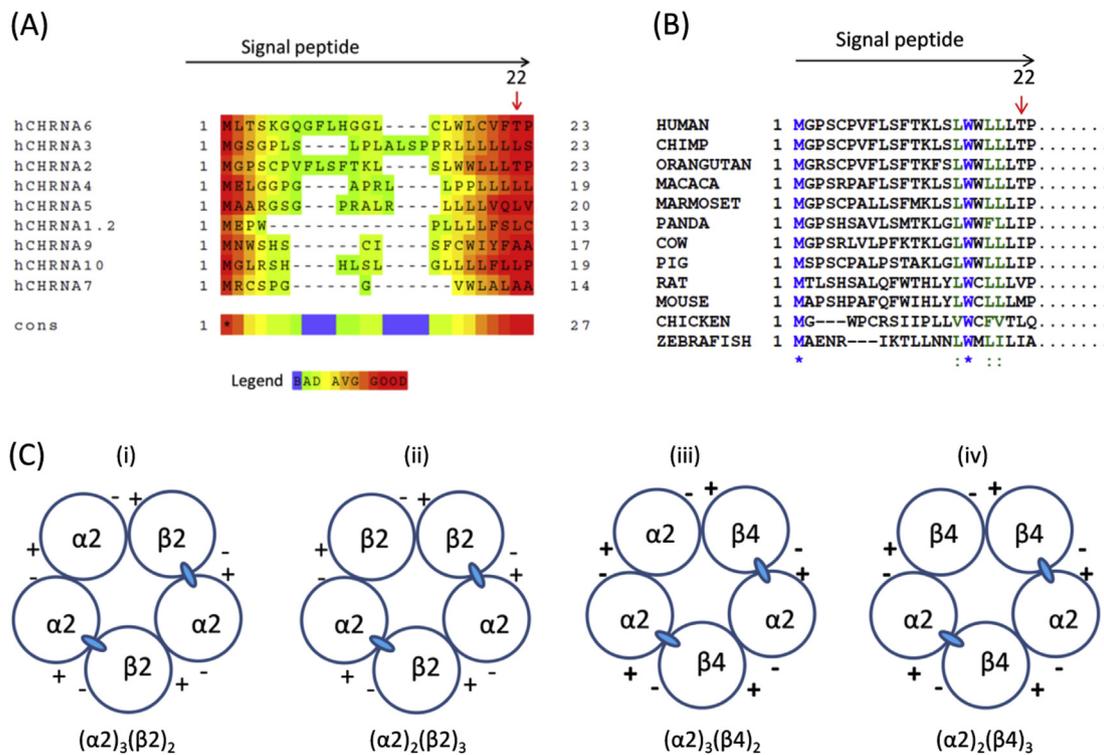
Signal peptide sequences of human nAChR  $\alpha$  ( $\alpha 1$ – $\alpha 10$ ) subunits or nAChR  $\alpha 2$  subunits from various organisms were aligned using Tcoffee (<http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee.cgi/index.cgi>) or ClustalW and portions of the alignments are presented (Fig. 1). Changes that might occur in the secondary structure characteristics of the nAChR  $\alpha 2$  SP due to the presence of an isoleucine residue (I) instead of a threonine (T) were assessed using several web-based protein prediction programs such as MINNOU (Membrane protein Identification withOUT explicit use of hydrophathy profiles and alignments) (<http://minnou.cchmc.org/>), Phobius (a combined transmembrane topology and signal peptide predictor) (<http://phobius.sbc.su.se/>), HHPred (Homology detection & structure prediction by HMM–HMM comparison) (<http://toolkit.tuebingen.mpg.de/hhpred>), and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>). Whether the change in nucleotide as a result of the SNP would lead to destruction or introduction of miRNA and/or other (snoRNAs and scaRNAs) RNA regulatory sites was evaluated by scanning the WT or mutant nucleotide sequences of human nAChR  $\alpha 2$  mRNA in miR-BASE ([www.mirbase.org](http://www.mirbase.org)), TargetScan ([www.targetscan.org](http://www.targetscan.org)), and snoRNA-LBME-db ([www.snorna.biotoul.fr](http://www.snorna.biotoul.fr)) registries.

### 2.2. Chemicals

All chemicals used in electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO, USA) except that L-nicotine was obtained from Arcos Organics (New Jersey, USA). Working solutions of acetylcholine (ACh), L-nicotine, atropine or mecamylamine were prepared daily in oocyte Ringer's solution (OR2) which consisted of (in mM) 92.5 NaCl, 2.5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 5 HEPES; and was adjusted to pH 7.5 by NaOH.

### 2.3. Subcloning, mutagenesis and in vitro transcription of nicotinic receptor subunits

Human nAChR  $\alpha 2$ ,  $\beta 2$  and  $\beta 4$  subunits were subcloned into the oocyte expression vector pGEMHE as earlier described (Dash et al., 2012). A synthetic, nAChR h $\beta 2$  subunit with nucleotide sequences optimized for better heterologous expression (h $\beta 2_{opt}$ ) was made (Invitrogen/GENEART, Burlingame, CA) and subcloned into the pCI vector (Promega, San Luis Obispo, CA) (Dash et al., 2011a; Dash and Lukas, 2012). There was not the necessity to codon optimize the nAChR h $\beta 4$  subunit as  $\alpha 2\beta 4$ -nAChR relatively highly functional. The mutation in the nAChR h $\alpha 2$  subunit was introduced in the pGEMHE background using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotides used for mutating threonine (T) to isoleucine (I) at nAChR  $\alpha 2$  subunit residue 22 are 5'-gtggctctctgaAcccagcaggtggag-3' (Forward, capitalization indicates the nucleotide changed from the wild-type) and 5'-ctccacctgctgggTcagaaggagccac-3' (Reverse). Identities of all wild-type (WT) or mutant subunits were confirmed by sequencing referenced to nucleotide/protein sequences available in GenBank.



**Fig. 1.** The threonine-to-isoleucine (T22I) mutation in the signal peptide (SP) of human nAChR  $\alpha 2$  subunit in relation to other nAChR alpha subunits and its potential effect on the probable stoichiometry's of  $\alpha 2^*$ -nAChR. (A) Sequence alignment of SP of human nAChR alpha subunits including that of nAChR  $\alpha 2$  subunit: SP sequences of human nAChR alpha subunits ( $\alpha 1$ – $\alpha 10$ ) are aligned using Toffee (<http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee.cgi/index.cgi>). Only a part of the alignment is shown here and the threonine (T) residue at position 22 [that has a mutant isoleucine (I) allele] in nAChR  $\alpha 2$  subunit is identified by an arrow mark. Because of the diversity of AA residues in the SP, the alignment may not be an optimal one. Also, an alternative alignment of the SP sequences of nAChR alpha subunits was done using ClustalW (data not shown). Both (Tcoffee and ClustalW) alignments identified the presence of the same or similar AA residues in position equivalent to AA 22 in nAChR  $\alpha 2$  subunit. (B) SP sequence alignment of nAChR  $\alpha 2$  subunits of various organisms: SP sequences of  $\alpha 2$ -nAChR subunits from several species [GenBank: NM\_000742.3 (Human: *Homo sapiens*), NM\_001033935.1 (Chimpanzee: *Pan troglodytes*), XM\_002818935.1 (Orangutan: *Pongo abelii*), XM\_001109335.2 (Monkey: *Macaca mulatta*), XM\_002756815.1 (Marmoset: *Callithrix jacchus*), XM\_002914435.1 (Panda: *Ailuropoda melanoleuca*), NM\_001192710.1 (Cow: *Bos taurus*), XM\_003132824.1 (Pig: *Sus scrofa*), NM\_133420.1 (Rat: *Rattus norvegicus*), NM\_144803.2 (Mouse: *Mus musculus*), NM\_204815.1 (Chicken: *Gallus gallus*) and NM\_001040327.1 (Zebrafish: *Danio rerio*)] were aligned using ClustalW after translating their mRNA or genomic sequences to protein sequences. Symbols below sequences indicate fully (\*) and strongly (:) conserved residues. For both (A) and (B), numbering begins at translation start methionine of nAChR subunit protein and is shown in the region of interest. (C) Schematic illustration of putative stoichiometries of wild-type (WT) or mutant  $\alpha 2^*$ -nAChR: Adhering to the canonical rule of pentamer formation,  $\alpha 2\beta 2$ -nAChR would be formed out of three  $\alpha 2$  and two  $\beta 2$  subunits (i) or two  $\alpha 2$  and three  $\beta 2$  subunits (ii) representing low sensitivity (LS) or high sensitivity (HS) nAChR respectively. In a similar fashion, putative low sensitivity (LS) or high sensitivity (HS)  $\alpha 2\beta 4$ -nAChR would be formed out of three  $\alpha 2$  and two  $\beta 4$  subunits (iii) or two  $\alpha 2$  and three  $\beta 4$  subunits (iv) Agonist (ACh or nicotine and others) binding sites in the interface of alpha and beta subunits are identified as ovals in all stoichiometries. Putative  $\alpha 2(T22I)^*$ -nAChR will attain such stoichiometries when  $\alpha 2(T22I)$  subunits substitute for WT  $\alpha 2$  subunit. It is hypothesized that the SP mutation associated with nicotine dependence would alter the stoichiometries of WT  $\alpha 2^*$ -nAChR.

All pGEMHE plasmids were linearized immediately downstream of the 3'-polyadenylation sequence. *NheI* was used to linearize nAChR  $\alpha 2$ ,  $\alpha 2(T22I)$  and  $\beta 4$  subunit-containing plasmids, and *SbfI* was used for linearizing WT  $\beta 2$  subunit-containing plasmids. *SwaI* was used to linearize  $\beta 2_{opt}$  subunit containing plasmids. Full length, capped complementary RNA (cRNA) was transcribed from linearized plasmids in a reaction mixture (20  $\mu$ L) using mMESAGE mMACHINE<sup>®</sup> T7 Kit (Invitrogen/Ambion Inc., CA, USA) and following the manufacturer's instructions. cRNA for all the WT and mutant nAChR subunits used in the current study were prepared in the same day using same batch of reagents. Integrity and quality of the cRNA was checked by electrophoresis and UV-spectroscopy.

#### 2.4. Dilution of cRNA for expression of $\alpha 2^*$ -nAChR

To express  $\alpha 2^*$ -nAChR in oocytes we planned to inject cRNA corresponding to each  $\alpha$  or  $\beta$  subunit in ratios of 1:1, 1:10 or 10:1. The 1:1 ratio approach more likely to result in expression of equal amounts of each subunit protein than the biased ratios (1:10 or 10:1) approaches. The latter kind of approaches has been proven to favor formation of high or low sensitivity (HS- or LS-) receptors,

respectively, defined based on their sensitivity to standard nicotinic agonists and thought to be formed from complexes containing 2:3 or 3:2 ratios of  $\alpha$ : $\beta$  subunits (Nelson et al., 2003; Zwart and Vijverberg, 1998). We exploited this approach, using 10:1 ratios for injection of cRNAs encoding  $\alpha 2$ : $\beta 2$ ,  $\alpha 2(T22I)$ : $\beta 2$ ,  $\alpha 2$ : $\beta 4$  or  $\alpha 2(T22I)$ : $\beta 4$  subunits to bias toward predominant expression of putative LS [( $\alpha 2$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub>-, ( $\alpha 2(T22I)$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub>-, ( $\alpha 2$ )<sub>3</sub>( $\beta 4$ )<sub>2</sub>- or ( $\alpha 2(T22I)$ )<sub>3</sub>( $\beta 4$ )<sub>2</sub>-] nAChR and using 1:10 ratios for injection of cRNA encoding  $\alpha 2$ : $\beta 2$ ,  $\alpha 2(T22I)$ : $\beta 2$ ,  $\alpha 2$ : $\beta 4$  or  $\alpha 2(T22I)$ : $\beta 4$  subunits to bias toward expression of putative HS [( $\alpha 2$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub>-, ( $\alpha 2(T22I)$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub>-, ( $\alpha 2$ )<sub>2</sub>( $\beta 4$ )<sub>3</sub>- or ( $\alpha 2(T22I)$ )<sub>2</sub>( $\beta 4$ )<sub>3</sub>-] nAChR (Fig. 1).

Concentration of cRNA for each nAChR  $\alpha$  and  $\beta$  subunits was initially adjusted to 777 ng/ $\mu$ L. Ten (10) fold further dilution of the cRNA for each  $\alpha$  and  $\beta$  subunits were made (i.e., final concentration of 77.7 ng/ $\mu$ L) for use in biased ratios ( $\alpha$ : $\beta$  ::10:1 or 1:10) studies. Several cRNA mixtures for each  $\alpha$ : $\beta$  subunit ratios were made (by mixing equal volume of cRNA for each  $\alpha$  and  $\beta$  subunit) and stored at  $-80^\circ\text{C}$  until further use. Injection of 69 nL cRNA mixture into each oocyte would deliver  $\sim 27$  ng of cRNA for each  $\alpha$  and  $\beta$  subunit for 1:1 ratio,  $\sim 27$  ng and  $\sim 2.7$  ng of cRNA for each  $\alpha$  and  $\beta$  subunits for 10:1 ratio or  $\sim 2.7$  ng and  $\sim 27$  ng of cRNA for each  $\alpha$  and  $\beta$  subunit for 1:10 ratio.

### 2.5. Oocyte preparation and cRNA injection

All experimental procedures were conducted in accordance with the guidelines of the National Institutes of Health (NIH) for the proper use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACU) of University of Virginia. Mature, female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI, USA) and were maintained in the University of Virginia Jordan Aquatic Facility until further use. Frogs were anesthetized using 0.2% tricaine methanesulfonate (MS-222) (Nasco, Fort Atkinson, WI, USA). Ovarian lobes were surgically removed from the frogs and placed in an incubation solution that consisted of (in mM) 82.5 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 1 Na<sub>2</sub>HPO<sub>4</sub>, 0.6 theophylline, 2.5 sodium pyruvate, 5 HEPES supplemented with 50 mg/ml gentamycin, 50 U/ml penicillin, 50 µg/ml streptomycin and adjusted to pH 7.5. Ovarian lobes were cut into small pieces and digested with 0.08 Wunsch U/ml liberase blendzyme 3 (Roche Applied Science, Indianapolis, IN) with constant shaking at room temperature for 1–1.5 h. The dispersed oocytes were thoroughly rinsed with incubation solution. Stage VI oocytes were selected and incubated at 16 °C before injection. Micropipettes used for injection were pulled from borosilicate glass (Drummond Scientific, Broomall, PA) using a Sutter P1000 horizontal puller (Sutter Instrument Company, Novato, CA, USA), and the tips were broken with forceps to ~40 µm in diameter. Four µL (4 µL) cRNA was drawn up into the micropipette and 69 nL cRNA was injected into each oocyte using a Nanoject II microinjection system (Drummond Scientific, Broomall, PA).

### 2.6. Oocyte electrophysiology

One to five days after injection, oocytes were placed in a small-volume chamber and continuously perfused with OR2. The chamber was grounded through an agarose bridge saturated with 3 M KCl. The oocytes were voltage-clamped at –70 mV to measure agonist-induced or antagonist-inhibited currents using Axoclamp 900A and pClamp 10.2 software (Axon Instruments/Molecular Devices, Sunnyvale, CA). The current signal was low-pass filtered at 10 Hz with the built-in low-pass Bessel filter in the Axoclamp 900A and digitized at 20 Hz with Axon Digidata1440A and pClamp 10.2. Electrodes contained 3 M KCl and had a resistance of 1–2 MΩ. Drugs (agonists and antagonists) were applied using a Valvelink 8.2 perfusion system (Automate scientific, Berkeley, CA). One micromolar (1 µM) atropine was always co-applied for acetylcholine (ACh)-based recordings to eliminate muscarinic receptor responses. Drug applications lasted for at least 5 s or noted otherwise. All electrophysiological measurements were conducted or checked in at least two batches of oocytes.

### 2.7. Experimental controls

Injection of water or empty vector (used as two forms of negative controls) or of cRNA corresponding to one subunit alone did not result in the expression of functional nAChR. Current responses to 100 µM nicotine or 100 µM ACh were less than 5–10 nA (data not shown).

### 2.8. Data analyses

Raw data was collected and processed in part using pClamp 10.2 (Molecular Devices, Sunnyvale, CA), Origin 7.5 (OriginLab Corporation, Northampton, MA) and a spreadsheet (Excel; Microsoft, Bellevue, WA), using peak current amplitudes as measures of functional nAChR expression. Data pooled across experiments are reported as mean ± SEM (for results from at least three oocytes, i.e.,  $n = 3$ ). Concentration–response (CR) relationships, in which mean peak current amplitudes at specified ligand concentrations were fit to the

Hill equation or its variants using Prism 4 (GraphPad Software, San Diego, CA), were constructed to assess true  $I_{max}$  (mean current amplitudes in response to the most efficacious concentration of an agonist) and  $EC_{50}$  (concentration for half-maximal activation) values.

$EC_{50}$  values for variant  $\alpha 2$ -nAChR was compared to that of the WT  $\alpha 2$ -nAChR expressed using comparable cRNA ratios. Also the effect of cRNA ratio variation on agonist  $EC_{50}$  values across WT or mutant  $\alpha 2$ -nAChR was assessed. The  $F$ -test ( $p < 0.05$  to define statistical significance) was used to compare the best fit values of log molar  $EC_{50}$  (concentration for half-maximal activation) values across specific nAChR subunit combinations.  $EC_{50}$  values with non-overlapping 95% confidence intervals (CI) deemed to be statistically significant ( $p < 0.05$ ).

There are limitations in the ability to compare peak current responses of nAChRs, even though we injected similar amounts of cRNAs for 1:1 coexpressions and biased (10:1 or 1:10) amount of cRNAs for LS- and HS- nAChR expressions, as we previously described previously (Dash et al., 2011a,b, 2012; Dash and Lukas, 2012). This is because expression levels assessed as peak current amplitudes are affected by batch-to-batch variation in oocytes, time between cRNA injection and recording, and subunit combination-specific parameters, such as open probability (influenced by gating rate constants, rates and extents of desensitization), single channel conductance, assembly efficiency, and efficiency of receptor trafficking to the cell surface (Groot-Kormelink et al., 2001). We made no attempt to measure or control for subunit combination-specific effects, but whenever preliminary studies revealed possible differences in peak current amplitudes, findings were further confirmed across different subunit combinations using the same batch of oocytes and the same time between cRNA injection and recording. However, when we make statements about results comparing ligand potencies and peak current amplitudes across subunit combinations, we do so for studies done under the same or very similar conditions, and the observations are clear, statistically significant, and in agreement whether for pooled data or for results from smaller sets of studies. The  $I_{max}$  values of the variant  $\alpha 2$ -nAChR was compared (Student's  $t$  test; two-tailed; \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ) to that of the WT  $\alpha 2$ -nAChR expressed using comparable cRNA ratios. The results of the study also warranted comparisons of  $I_{max}$  values (one-way ANOVA with Tukey's post hoc comparison: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$ ) of the WT  $\alpha 2$ -nAChR across different cRNA ratios and also within variant  $\alpha 2$ -nAChR expressed using different cRNA ratios to assess their relative functionality.

## 3. Results

### 3.1. In silico analysis of T22I mutation in human nAChR $\alpha 2$ subunit indicated possible functional consequences for such a mutation

The degree of evolutionary conservation of a threonine or isoleucine residue at amino acid (AA) position 22 in the nAChR  $\alpha 2$  subunit was explored among other human nAChR  $\alpha$  subunits and nAChR  $\alpha 2$  subunits from several other organisms (Fig. 1). Threonine (nAChR  $\alpha 4$  and  $\alpha 6$ ), alanine (nAChR  $\alpha 7$  and  $\alpha 9$ ) or leucine (nAChR  $\alpha 5$ ,  $\alpha 3$ ,  $\alpha 10$  and  $\alpha 1$ ) residues are present at positions equivalent to AA 22 in the nAChR  $\alpha 2$  subunit. Other organisms have threonine (T), isoleucine (I), valine (V), methionine (M) or leucine (L) residue(s) at the same locus. These alignments indicated preference for a hydrophobic rather than a hydrophilic residue at AA 22. Secondary structure prediction analyses indicated possible alteration of a coil or alpha helix in the SP as a result of the mutation (Fig. S1; see supplementary information). PSIPRED (Fig. S1) and Phobius (data not shown) predicted that T22<sub>h22</sub> would be part of coil region immediately following a helix region (AA 14–21) in the SP, whereas I22<sub>h22</sub> would be incorporated into the helical region. MINNOU predicted

that either T22<sub>hα2</sub> or I22<sub>hα2</sub> at position 22 would be part of a 2 residue β-strand or bridge that is preceded by a helical structure. Nonetheless, since alpha helices in signal peptides (generally rich in hydrophobic residues) are important for anchoring protein molecules to membranes, substitution of a hydrophobic isoleucine for hydrophilic threonine that probably alters the secondary structure of the SP could have functional consequences.

3.2. Expression of a codon-optimized human nAChR β2 subunit (hβ2<sub>opt</sub>) enhances the agonist induced currents of human α2β2-nAChR

Generally, coexpression of WT nAChR hα2 and hβ2 subunits in *Xenopus* oocytes does not lead to expression of highly functional hα2hβ2-nAChR (Dash et al., 2012). However, coinjection of similar quantities (ng) of cRNAs for nAChR hα3 and hβ2 nAChR subunits or hα4 and hβ2 subunits lead to highly functional hα3hβ2- or hα4hβ2-nAChR, respectively (Dash et al., 2012). In order to achieve expression of several hundred nanoamperes (nA) of peak current by agonist-activated hα2hβ2-nAChR, a codon-optimized human nAChR β2 subunit (hβ2<sub>opt</sub>) (<http://www.ncbi.nlm.nih.gov/nuccore/JN565027>) was coexpressed with hα2 subunits. Advantages of use of such codon-optimized nAChR subunits were demonstrated previously (Dash et al., 2011b; Dash and Lukas, 2012; Slimko and Lester, 2003). Results indicated that receptors expressed in oocytes injected with hα2 and hβ2<sub>opt</sub> subunit cRNAs elicit several fold higher (*p* < 0.05) peak current than those expressed in oocytes injected with hα2 and hβ2<sub>wt</sub> subunit cRNAs when activated by either 316 μM ACh (hα2hβ2<sub>wt</sub> vs. hα2hβ2<sub>opt</sub>: 28 ± 6 nA vs. 406 ± 89 nA; 14-fold increase; *p* < 0.01) or 100 μM nicotine (hα2hβ2<sub>wt</sub> vs. hα2hβ2<sub>opt</sub>: 24 ± 5 nA vs. 291 ± 55 nA; 12-fold increase; *p* < 0.05). Hence cRNA for the codon-optimized nAChR hβ2 subunit was used in subsequent hα2hβ2-nAChR expression studies.

All hα2hβ2-nAChR function subsequently noted is for receptors containing hβ2<sub>opt</sub> subunits. A codon optimized version of the human nAChR β4 subunit was not used in this study as hα2hβ4-nAChRs expressed in oocytes are relatively highly functional.

3.3. The T22I mutation in the nAChR α2 subunit alters function of α2β2-nAChR

3.3.1. Expression of WT or mutant α2β2-nAChRs in oocytes injected with a 1:1 ratio of α2(WT or mutant):β2 subunit cRNAs

Human nAChRs were assembled in *Xenopus* oocytes after injection of a mixture in equal quantities (1:1) of cRNAs encoding α2 and β2 subunits or α2(T22I) and β2 subunits and were functionally activated by ACh or nicotine (Figs. 2 and 3 and S2; Table 1). Concentration-response (CR) relationships yielded EC<sub>50</sub> values (concentrations for half-maximal activation) indicating lower potencies (*p* < 0.0001) for both ACh and nicotine (~4-fold) acting at α2(T22I)β2-nAChR than acting at WT α2β2-nAChR (Table 1). Mutant α2(T22I) subunit inclusion instead of WT α2 subunits produced receptors with increased I<sub>max</sub> (*p* < 0.001) for exposure to 316 μM ACh or 100 μM nicotine (Fig. 2(B); Table 1).

These results observed could be due to responses of a mixture of LS- and HS- α2\*-nAChRs expressed in oocytes postulated to have 3:2 and 2:3 ratios, respectively, of α:β subunits as has been the case with α4β2-nAChR (Nelson et al., 2003). Thus, we conducted additional experiments by manipulating subunit cRNA ratios for expression of putative LS- or HS- {α2 or α2(T22I)}β2- nAChR in oocytes.

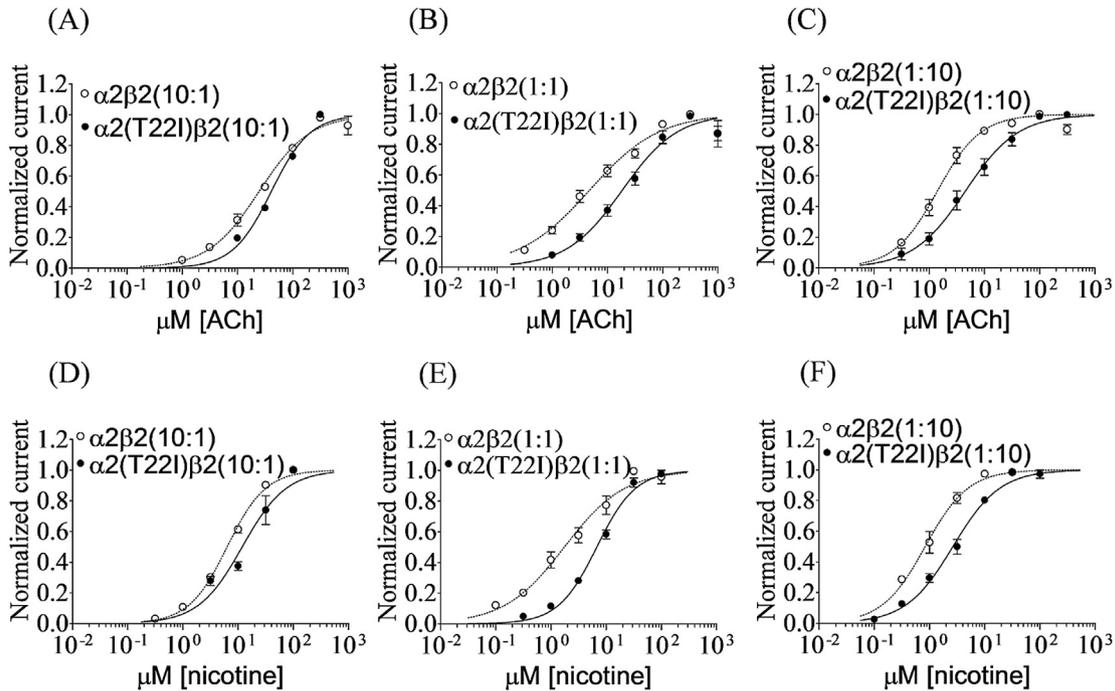
3.3.2. Expression of putative low sensitivity (LS) WT or mutant α2β2-nAChR in oocytes injected with a 10:1 ratio of α2(WT or mutant):β2 subunit cRNAs

As expected, receptors expressed in oocytes injected with a 10:1 ratio of α2:β2 subunit cRNAs displayed lower (*p* < 0.001) ACh (~5

Table 1

Parameters for drug action at human α2β2-, α2(T22I)β2-, α2β4- and α2(T22I)β4- nAChR. Potencies (micromolar EC<sub>50</sub> values and 95% confidence intervals), Hill coefficients (n<sub>H</sub> ± SEM), average (±SEM) peak response (nA), and the concentration where I<sub>max</sub> is achieved (μM) are provided for the indicated agonist (ACh or nicotine) acting at nAChR composed of the indicated subunits and from the indicated number of independent experiments based on studies as shown in Figs. 2–5. ↓ or ↑ indicates a significant (*p* < 0.05) decrease or increase, respectively, in the relevant parameter for nAChR containing α2(T22I) instead of wild-type α2 subunits. Numeric x indicates fold change.

Agonist	nAChR subunit combination	Potency			Peak response		
		n	EC <sub>50</sub> (μM) (95% CI)	n <sub>H</sub> ± SE	n	I <sub>max</sub> (nA) (Mean ± SE)	I <sub>max</sub> conc. (μM)
ACh	α2β2(10:1)	5	25 (21–30)	0.9 ± 0.08	11	314 ± 84	316
	α2β2(1:1)	8	4.7 (3.8–5.9)	0.7 ± 0.05	26	551 ± 62	316
	α2β2(1:10)	6	1.4 (1.2–1.7)	1 ± 0.08	18	167 ± 18	100
	α2(T22I)β2(10:1)	3	41 (21–88) ↑1.6x	0.9 ± 0.3	6	54 ± 9 ↓5.8x	316
	α2(T22I)β2(1:1)	8	19 (14–24) ↑4x	0.9 ± 0.08	14	1326 ± 214 ↑2.4x	316
	α2(T22I)β2(1:10)	6	4.5 (3.6–5.7) ↑3.2x	0.9 ± 0.09	7	952 ± 128 ↑5.7x	100
Nicotine	α2β2(10:1)	3	6.3 (5.8–6.9)	1.2 ± 0.06	15	239 ± 21	100
	α2β2(1:1)	5	1.8 (1.4–2.3)	0.8 ± 0.08	14	373 ± 33	100
	α2β2(1:10)	5	0.83 (0.67–1)	1.1 ± 0.13	11	68 ± 7.8	31.6
	α2(T22I)β2(10:1)	3	12 (7.5–19) ↑1.9x	1.1 ± 0.2	13	62 ± 12 ↓3.8x	100
	α2(T22I)β2(1:1)	6	6.7 (6–7.6) ↑3.7x	1.2 ± 0.08	21	1463 ± 136 ↑3.9x	100
	α2(T22I)β2(1:10)	6	2.6 (2.3–3.1) ↑3.1x	1 ± 0.07	12	1004 ± 91 ↑14.8x	31.6
ACh	α2β4(10:1)	7	40 (37–43)	1.5 ± 0.07	18	1068 ± 113	316
	α2β4(1:1)	7	15 (13–17)	1.2 ± 0.08	17	2391 ± 150	316
	α2β4(1:10)	5	10 (8.6–12)	1.1 ± 0.09	21	2405 ± 173	316
	α2(T22I)β4(10:1)	3	48 (42–55)	1.9 ± 0.2	10	337 ± 51 ↓3.2x	316
	α2(T22I)β4(1:1)	5	36 (32–41) ↑2.4x	1.5 ± 0.1	14	2019 ± 167 ↓1.2x	316
	α2(T22I)β4(1:10)	4	4.3 (3.6–5.2) ↓2.3x	1.1 ± 0.09	12	4676 ± 130 ↑1.9x	316
Nicotine	α2β4(10:1)	3	11 (10–12)	1.6 ± 0.09	21	1139 ± 93	100
	α2β4(1:1)	4	4.7 (3.6–6.1)	1.1 ± 0.1	19	2088 ± 96	100
	α2β4(1:10)	4	3.8 (3.4–4.3)	1.3 ± 0.08	24	1814 ± 155	100
	α2(T22I)β4(10:1)	6	14 (13–16)	2 ± 0.2	16	402 ± 70 ↓2.8x	100
	α2(T22I)β4(1:1)	7	12 (11–13) ↑2.6x	1.8 ± 0.07	17	1527 ± 245 ↓1.4x	100
	α2(T22I)β4(1:10)	6	2.6 (2.4–2.7) ↓1.5x	1.2 ± 0.04	16	3996 ± 168 ↑2.2x	100



**Fig. 2.** Comparison of concentration–response (CR) relationships between WT  $\alpha 2\beta 2$ - and mutant  $\alpha 2(T221)\beta 2$ -nAChR expressed in oocytes injected with 10:1, 1:1 or 1:10 subunit cRNAs. Results averaged across experiments were used to produce CR curves (ordinate – mean normalized current  $\pm$  SEM; abscissa – ligand concentration in log  $\mu$ M) for  $\alpha 2\beta 2$ - ( $\circ$ ) or  $\alpha 2(T221)\beta 2$ - ( $\bullet$ ) nAChR expressed in oocytes injected with 10:1 (A and D), 1:1 (B and E) or 1:10 (C and F) ratios of subunit cRNAs for their responses to ACh [A, B and C] or nicotine [D, E and F] as indicated. Rightward shifts in agonist CR curves are evident for  $\alpha 2(T221)\beta 2$ -nAChR. Parameters for agonist action are summarized in Table 1. See Fig. S2(A) for comparisons of CR relationships within the 3 isoforms of  $\alpha 2\beta 2$ - or  $\alpha 2(T221)\beta 2$ -nAChR.

fold) or nicotine ( $\sim 3.5$  fold) sensitivity than those expressed in oocytes injected with a 1:1 ratio of  $\alpha 2$ : $\beta 2$  subunit cRNAs (Fig. S2; Table 1). Also, oocytes expressing presumed  $\{\alpha 2(T221)\}_3(\beta 2)_2$ -nAChR displayed generally lower ACh ( $\sim 2$ -fold) or nicotine ( $\sim 2$ -fold) sensitivity than those expressed in oocytes injected with a 1:1  $\alpha 2(T221)$ : $\beta 2$  subunit cRNA (Fig. S2; Table 1). Together these results indicated a lower potency for ACh ( $\sim 1.6$ -fold) or nicotine ( $\sim 2$ -fold,  $p < 0.0001$ ) acting at  $\alpha 2(T221)\beta 2$ - than acting at  $\alpha 2\beta 2$ -nAChR expressed in oocytes injected with a 10:1 ratio of  $\alpha$ : $\beta$  subunit cRNAs (Fig. 2; Table 1).

nAChRs expressed in oocytes injected with  $\alpha 2$  and  $\beta 2$  cRNAs in a 10:1 ratio had lower  $I_{max}$  ( $\sim 43\%$  for ACh,  $p < 0.05$ ;  $\sim 36\%$  for nicotine,  $p < 0.001$ ) than receptors expressed in oocytes injected with a 1:1 ratio of  $\alpha 2$  and  $\beta 2$  subunit cRNAs (Fig. 3; Table 1). Similarly, receptors expressed in oocytes injected with  $\alpha 2(T221)$  and  $\beta 2$  cRNAs in a 10:1 ratio had lower ( $p < 0.001$ )  $I_{max}$  in response to ACh ( $\sim 25$ -fold) or nicotine ( $\sim 23$  fold) than nAChR expressed in oocytes injected with a 1:1 ratio of  $\alpha 2(T221)$  and  $\beta 2$  subunit cRNAs (Fig. 3; Table 1). Hence, in oocytes injected at 10:1  $\alpha$ : $\beta$  subunit cRNA ratios, presumed  $\{\alpha 2(T221)\}_3(\beta 2)_2$ -nAChR had lower  $I_{max}$  to ACh ( $\sim 6$  fold) or nicotine ( $\sim 4$ -fold) than did presumed  $(\alpha 2)_3(\beta 2)_2$ -nAChR (Table 1).

### 3.3.3. Expression of putative high sensitivity (HS) WT or mutant $\alpha 2\beta 2$ -nAChR in oocytes injected with a 1:10 ratio of $\alpha 2$ (WT or mutant): $\beta 2$ subunit cRNAs

Receptors expressed in oocytes injected with a 10-fold excess of  $\beta 2$  over  $\alpha 2$  subunit (i.e., HS- $\alpha 2\beta 2$ -nAChR) cRNAs displayed higher ( $p < 0.001$ ) ACh or nicotine sensitivity than those expressed in oocytes injected with 1:1 (mixture of LS and HS isoforms;  $\sim 2$ –3 fold) or 10:1 (LS;  $\sim 8$ –18-fold) subunit cRNAs (Fig. S2; Table 1). Similarly mutant HS [i.e.,  $\{\alpha 2(T221)\}_2(\beta 2)_3$ -] nAChR expressed in

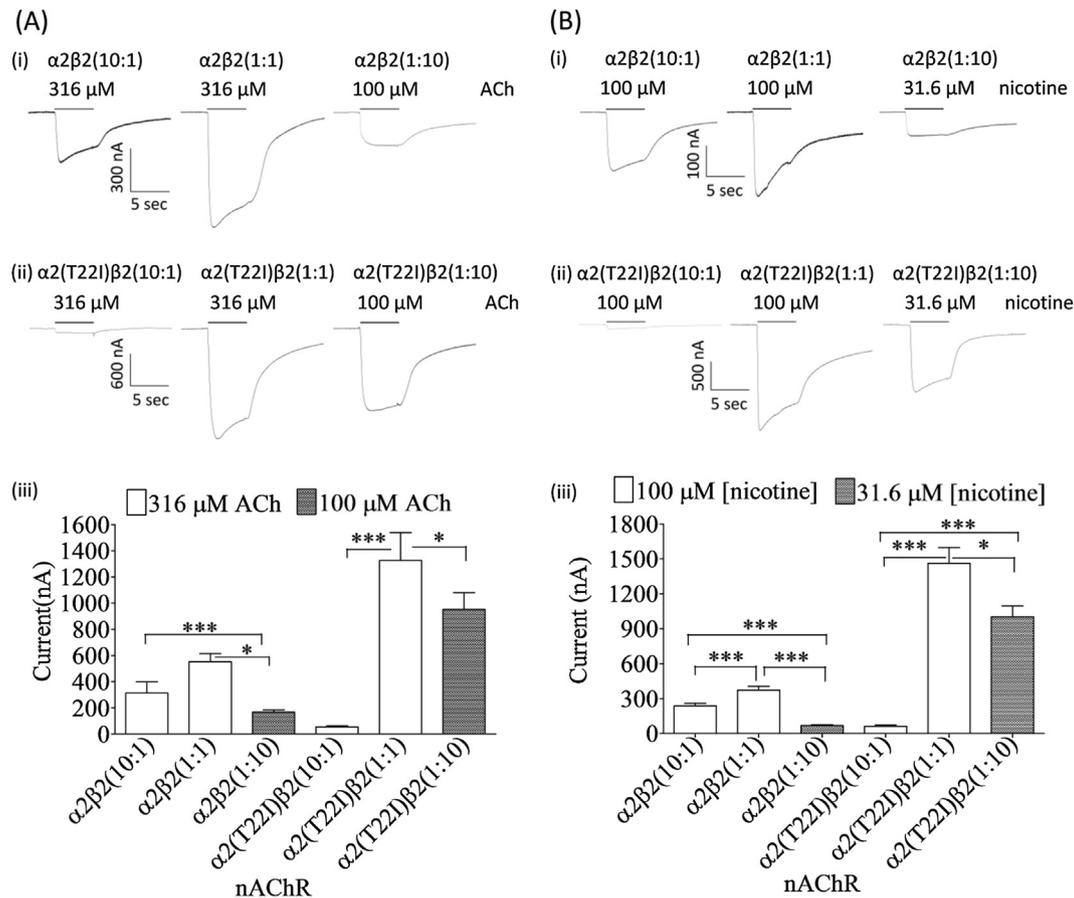
oocytes injected with a 1:10 ratios of  $\alpha 2(T221)$ : $\beta 2$  subunit cRNAs displayed higher ( $p < 0.001$ ) ACh ( $\sim 4$ –9 fold) or nicotine ( $\sim 3$ –5-fold) sensitivity than those expressed with 1:1 (mixture of isoforms) or 10:1 (LS) subunit cRNAs (Fig. S2; Table 1). These results also indicated a lower ( $p < 0.001$ ) potency for ACh or nicotine ( $\sim 3$ -fold) acting at  $\alpha 2(T221)\beta 2$ -nAChR than acting at WT  $\alpha 2\beta 2$ -nAChR when receptors are expressed in oocytes injected with 1:10  $\alpha$ : $\beta$  subunit cRNAs (Fig. 2; Table 1).

$I_{max}$  for receptors expressed in oocytes injected with a 1:10 ratio of nAChR  $\alpha 2$  and  $\beta 2$  subunit cRNAs (i.e., HS- $\alpha 2\beta 2$ -nAChR) generally tended lower ( $\sim 47$ –70% for ACh;  $\sim 72$ –82% for nicotine) for responses to agonists than for those expressed in oocytes injected with a 1:1 (mixture of isoforms) or 10:1 (LS) ratio of nAChR  $\alpha 2$  and  $\beta 2$  subunit cRNAs (Fig. 3; Table 1). However, agonist induced  $I_{max}$  of putative HS- $\alpha 2(T221)\beta 2$ -nAChRs [i.e.,  $\alpha 2(T221)$ : $\beta 2$ ::1:10] expressed in oocytes was higher ( $p < 0.001$ ) than those of putative LS- $\alpha 2(T221)\beta 2$ -nAChRs [i.e.,  $\alpha 2(T221)$ : $\beta 2$ ::10:1] but generally lower than (952 nA vs. 1326 nA for ACh;  $p > 0.05$ ) those nAChRs expressed injected with 1:10 ratio of nAChR.

For receptors expressed in oocytes injected with 1:10  $\alpha 2(T221)$ : $\beta 2$  subunit cRNAs, responses to 100  $\mu$ M ACh ( $\sim 6$  fold higher) or 31.6  $\mu$ M nicotine ( $\sim 15$  fold higher) were higher ( $p < 0.001$ ) than those expressed in oocytes injected with 1:10  $\alpha 2$ : $\beta 2$  subunit cRNAs (Fig. 3; Table 1).

### 3.4. The T221 mutation in the nAChR $\alpha 2$ subunit alters function of $\alpha 2\beta 4$ -nAChR

Studies also were done of human nAChRs assembled in oocytes injected with cRNAs encoding  $\alpha 2$  and  $\beta 4$  subunits or  $\alpha 2(T221)$  and  $\beta 4$  subunits in equal quantities (i.e., 1:1, presumably forming a mixture of receptor isoforms), at a 10:1 ratio [presumably favoring



**Fig. 3.** Current responses of WT  $\alpha 2\beta 2$ - and mutant  $\alpha 2(T221)\beta 2$ - nAChR to nicotinic ligands expressed in oocytes injected with 10:1, 1:1 or 1:10 subunit cRNAs. Representative traces are shown for inward currents in oocytes held at  $-70$  mV, responding to application of the indicated (most efficacious) concentrations of ACh [(A) (i) and (ii)] or nicotine [(B) (i) and (ii)] (shown with the duration of agonist exposure as black bars above the traces), and expressing indicated WT or mutant  $\alpha 2^*$ -nAChR. Calibration bars are for 300 or 600 nA (A), or 100 or 500 nA (B) currents (vertical) and 5 s (horizontal). Mean ( $\pm$ SEM) peak inward current responses from oocytes ( $n = 6-26$ ; see Table 1 for details) to indicated concentration of ACh [(A) (iii)] or nicotine [(B) (iii)] were compared among groups of  $\alpha 2\beta 2$ - or  $\alpha 2(T221)\beta 2$ - nAChR using one-way ANOVA with Tukey's post hoc comparison (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ). See Table 1 for comparisons between relevant stoichiometries of  $\alpha 2\beta 2$ - and  $\alpha 2(T221)\beta 2$ - nAChR.

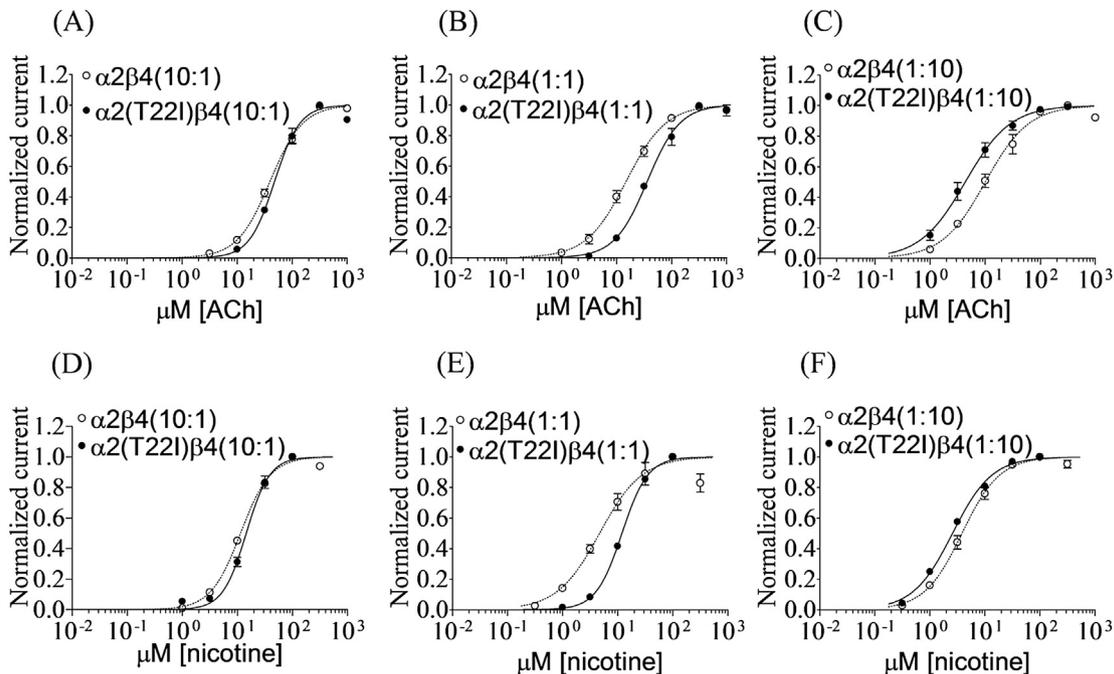
formation of LS [ $\alpha 2$  or  $\alpha 2(T221)$ ] $_3$ ( $\beta 2$ ) $_2$ -nAChR], or at a 1:10 ratio [presumably favoring formation of HS [ $\alpha 2$  or  $\alpha 2(T221)$ ] $_2$ ( $\beta 2$ ) $_3$ -nAChR]. All of these nAChR were functionally activated by ACh or nicotine (Figs. 4 and 5 and S2; Table 1). CR relationships yielded, as the  $\alpha 2$ : $\beta 4$  subunit message ratio decreased, the expected, progressive increase in sensitivity to ACh and to nicotine (Fig. S2).  $EC_{50}$  values for ACh were 40, 15 and 10  $\mu$ M for LS, 1:1 and HS  $\alpha 2\beta 4$ -nAChR, respectively ( $p < 0.001$  for 1:1 or HS compared to LS;  $p < 0.01$  for 1:1 compared to HS) and were 48, 36 and 4.3  $\mu$ M for LS, 1:1 and HS  $\alpha 2(T221)\beta 4$ -nAChR, respectively ( $p < 0.01$  for 1:1 compared to LS;  $p < 0.001$  for LS or 1:1 compared to HS) (Fig. S2; Table 1).  $EC_{50}$  values for nicotine were 11, 4.7 and 3.8  $\mu$ M for LS, 1:1 and HS  $\alpha 2\beta 4$ -nAChRs, respectively ( $p < 0.001$  for 1:1 or HS compared to LS) and were 14, 12 and 2.6  $\mu$ M for LS, 1:1 and HS  $\alpha 2(T221)\beta 4$ -nAChRs, respectively ( $p < 0.01$  for LS or 1:1 compared to HS) (Fig. S2; Table 1). Moreover, Hill coefficients also decreased as the  $\alpha 2$ : $\beta 4$  subunit message ratios decreased (Table 1). Comparison of agonist potencies also reveals  $\sim 2-3$ -fold lower potencies ( $p < 0.001$ ; higher  $EC_{50}$  values) for both ACh and nicotine acting at  $\alpha 2(T221)\beta 4$ - than acting at  $\alpha 2\beta 4$ -nAChR when the mixture of HS and LS isoforms are expressed (Fig. 4; Table 1). Interestingly, agonist potencies were not significantly altered ( $p > 0.05$ ) when mutant  $\alpha 2$  subunits were substituted for WT  $\alpha 2$  subunits in excess over  $\beta 4$  subunits (LS; 48 vs. 40  $\mu$ M for ACh, 14 vs. 11  $\mu$ M for nicotine), but

agonist potencies were  $\sim 2$ -fold higher ( $p < 0.001$ ) for HS  $\alpha 2(T221)\beta 4$ -nAChR than for HS  $\alpha 2\beta 4$ -nAChR [1:10  $\alpha 2/\alpha 2(T221)$ : $\beta 4$  injected cRNA ratios].

$I_{max}$  concentrations for  $\alpha 2\beta 4$ - or  $\alpha 2(T221)\beta 4$ -nAChR were 316  $\mu$ M for ACh and 100  $\mu$ M for nicotine regardless of subunit ratios (Fig. 5; Table 1).  $I_{max}$  responses to either ACh or nicotine were highest for lower  $\alpha 2$ : $\beta 4$  or  $\alpha 2(T221)$ : $\beta 4$  subunit ratios ( $p < 0.001$  for 1:1 or HS compared to LS) (Fig. 5; Table 1). Substitution of  $\alpha 2(T221)$  for WT  $\alpha 2$  subunits lowered ( $p < 0.001$ ) peak current responses to agonists by about 3-fold for putative LS receptors, had modest effects for the mixture of receptor isoforms ( $\sim 15\%$  lower, 2019 vs. 2391 nA for ACh,  $p > 0.05$ ;  $\sim 30\%$  lower, 1527 vs. 2088 nA for nicotine;  $p < 0.05$ ), but approximately doubled ( $p < 0.001$ ) peak current amplitude when putative HS receptors were expressed in the presence of  $\beta 4$  over  $\alpha 2$  or  $\alpha 2(T221)$  subunits (Fig. 5; Table 1).

### 3.5. Mecamylamine blocks WT or mutant $\alpha 2(\beta 2$ or $\beta 4)$ -nAChR

Cognizant of the fact that mutant  $\alpha 2(T221)(\beta 2$  or  $\beta 4)$ -nAChRs are functionally different from that of WT  $\alpha 2(\beta 2$  or  $\beta 4)$ -nAChRs we wanted to know whether mecamylamine would block the mutant  $\alpha 2^*$ -nAChRs. Mecamylamine at 1000  $\mu$ M inhibited ACh-induced peak current responses of receptors expressed in oocytes injected with 10:1, 1:1 or 1:10  $\alpha 2$ : $\beta 4$  subunit cRNAs by 96%, 93% or 92%,



**Fig. 4.** Comparison of CR relationships of between WT  $\alpha 2\beta 4$ - and mutant  $\alpha 2(T22I)\beta 4$ -nAChR. Results averaged across experiments were used to produce CR curves (ordinate – mean normalized current  $\pm$  SEM; abscissa – ligand concentration in log  $\mu\text{M}$ ) for WT  $\alpha 2\beta 4$ - ( $\circ$ ) or mutant  $\alpha 2(T22I)\beta 4$ - ( $\bullet$ ) nAChR expressed in oocytes injected with 10:1 (A and D), 1:1 (B and E) or 1:10 (C and F) ratios of subunit cRNAs for their responses to ACh [A, B and C] or nicotine [D, E and F] as indicated. Rightward shifts in agonist CR curves for  $\alpha 2(T22I)\beta 4$ -nAChR expressed in oocytes injected with 1:1 or 10:1 ratio of subunit cRNAs and a leftward shift in agonist CR curves for  $\alpha 2(T22I)\beta 4$ -nAChR expressed in oocytes injected with 1:10 ratio of subunit cRNAs are evident. Parameters for agonist action are summarized in Table 1. See Fig. S2(B) for comparisons of CR relationships within the 3 isoforms of  $\alpha 2\beta 4$ - or  $\alpha 2(T22I)\beta 4$ -nAChR.

respectively (Fig. 6(A)). Also ACh-induced peak responses of  $\alpha 2(T22I)\beta 4$  receptors expressed in oocytes injected with 10:1, 1:1 or 1:10  $\alpha 2(T22I)\beta 4$  subunit cRNAs were inhibited by 1000  $\mu\text{M}$  mecamylamine by 99%, 99% or 83%, respectively (Fig. 6(B)). Similar degrees of inhibition were observed for mecamylamine effects on nicotine-induced peak responses of receptors expressed in oocytes injected with 10:1, 1:1 or 1:10 WT or mutant  $\{\alpha 2$  or  $\alpha 2(T22I)\}\beta 4$  subunit cRNAs (Fig. 6(C) and (D)). Mecamylamine at 1000  $\mu\text{M}$  completely abolished the ACh or nicotine induced  $I_{\text{max}}$  responses of receptors expressed in oocytes injected with 1:1, 10:1 or 1:10  $\alpha 2:\beta 2$  or  $\alpha 2(T22I):\beta 2$  subunit cRNAs (data not shown).

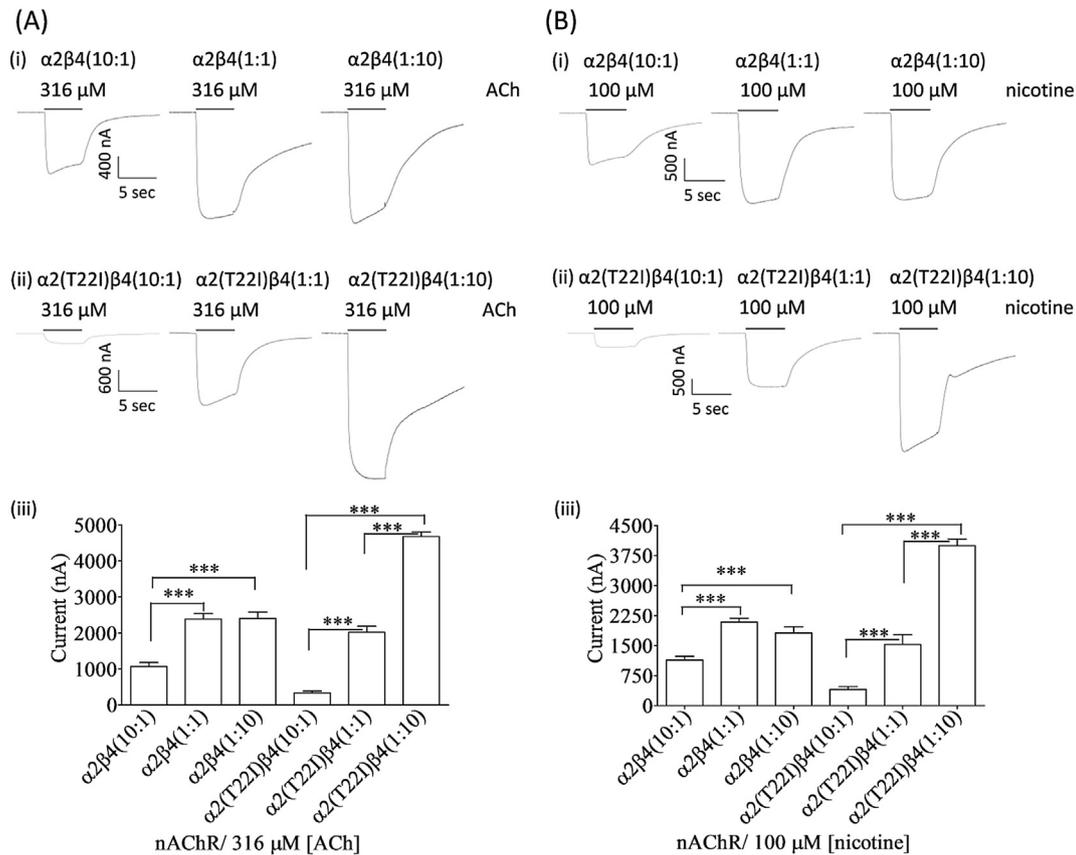
#### 4. Discussion

Several mutations in human nAChR subunits (Engel and Sine, 2005; Falvella et al., 2010; Haller et al., 2012; Tammimaki et al., 2012; Xie et al., 2011) having pathophysiological consequences have been characterized functionally. Most prominent among them are mutations in muscle nAChR subunits that lead to congenital myasthenic syndromes (CMS) (Engel and Sine, 2005), nAChR transmembrane II channel lining mutations associated with ADNFLE (Steinlein and Bertrand, 2008), and a mutation in nAChR  $\alpha 5$  subunit (D398N) (George et al., 2012; Kuryatov et al., 2011; Li et al., 2011; Tammimaki et al., 2012) associated with various diseases including ND. The T22I mutation in the nAChR  $\alpha 2$  subunit drew our attention because it has been associated with increased susceptibility to ND. Current results show effects of such a mutation on the function of  $\alpha 2^*$ -nAChR. These results also suggest that the effects of the SP mutation (despite being in a region that would be expected to be eliminated in the mature protein, at least in mammals) could be captured in oocyte expression system. The T22I mutation in nAChR  $\alpha 2$  subunit also is the first SP mutation in a member of cys-

loop family of ligand-gated ion channels (LGICs) to have functional consequences.

SP mutations have a variety of consequences of (Mencarelli et al., 2012; Mohren and Weiskirchen, 2009; Mukherjee et al., 2006) but these are not known for cys-loop receptors. For example, a SP mutation in the proopiomelanocortin (POMC) gene inhibits the production and then the overall secretion of POMC protein (Mencarelli et al., 2012). A non-synonymous gene polymorphism in the SP of human TGF- $\beta 1$  severely impairs protein synthesis (Mohren and Weiskirchen, 2009). As for the reported TGF- $\beta 1$  mutation, folding prediction analysis indicated a different RNA secondary structure for the mutant (A15G) POMC mRNA. These findings also indicate that SP mutations can critically compromise mRNA accessibility to the translation machinery, resulting in the absence of the protein product. In another study (Mukherjee et al., 2006), a missense mutation in the SP of progranulin is shown to cause hereditary dysphasic disinhibition dementia 2 (HDDD2), a familial frontotemporal lobar degeneration with ubiquitin-positive, tau-negative inclusions. Also the authors (Mukherjee et al., 2006) in light of the previous reports of null mutations and its position in the gene speculated two possible pathological mechanisms: (1) that the protein may accumulate within the endoplasmic reticulum due to inefficient secretion; and (2) that mutant RNA may have a lower expression because of degradation via nonsense-mediated decay. In light of these studies, it is tempting to propose that the T22I mutation in the SP of the nAChR  $\alpha 2$  gene could affect the processing and sorting of the nAChR  $\alpha 2$  protein to the regulated secretory pathway.

There could be many reasons for the intriguing observations made here. There is the possibility, as the bioinformatics analyses suggest, of alterations in nAChR  $\alpha 2$  subunit protein/SP structure. Therefore there is the formal possibility that structural alterations



**Fig. 5.** Current responses of WT  $\alpha 2\beta 4$ - and mutant  $\alpha 2(T221)\beta 4$ - nAChR to nicotinic ligands expressed in oocytes injected with 10:1, 1:1 or 1:10 subunit cRNAs. Representative traces are shown for inward currents in oocytes held at  $-70$  mV, responding to application at the indicated (most efficacious) concentrations of ACh [(A) (i) and (ii)] or nicotine [(B) (i) and (ii)] (shown with the duration of agonist exposure as black bars above the traces), and expressing indicated WT or mutant  $\alpha 2^*$ -nAChR. Calibration bars are for 400 or 600 nA (A), or 500 nA (B) currents (vertical) and 5 s (horizontal). Mean ( $\pm$ SEM) peak inward current responses from oocytes ( $n = 10$ – $24$ ; see Table 1 for details) to indicated concentration of ACh [(A) (iii)] or nicotine [(B) (iii)] were compared among groups of  $\alpha 2\beta 4$ - or  $\alpha 2(T221)\beta 4$ - nAChR using one-way ANOVA with Tukey's post hoc comparison (\*\*\*,  $p < 0.001$ ). See Table 1 for comparisons between relevant stoichiometries of WT  $\alpha 2\beta 4$ - and mutant  $\alpha 2(T221)\beta 4$ - nAChR.

imposed by the SP mutation could have different effects on agonist sensitivity of LS and HS  $\alpha 2(\beta 2$  or  $\beta 4)$ -nAChR. The mutation could alter SP cleavage, and differences in WT compared to mutant subunit RNA/protein secondary structure could affect efficiency of transcription, translation, assembly of subunits or receptor cell surface expression. It is possible that the T221 mutant subunits are not very efficient in assembling as a  $\alpha 2(T221)_3$ -nAChR or the trafficking of  $\alpha 2(T221)_3$ -nAChR is impaired. These conditions may lead to reduction in peak current responses (2.8–5.8-fold) and/or agonist potencies (1.6–1.9 fold) at mutant LS [i.e.,  $\alpha 2(T221)_3(\beta 2$  or  $\beta 4)_2$ -] nAChRs relative to those of WT nAChRs. Conversely two mutant  $\alpha 2(T221)$  subunits assemble with three nAChR  $\beta 2$  or  $\beta 4$  subunits efficiently or the HS [i.e.,  $\alpha 2(T221)_2(\beta 2$  or  $\beta 4)_3$ -] nAChR traffick efficiently to the cell surface leading to increase in peak current responses (1.9–15 fold) and the accompanying change in agonist potencies (3.1–3.2 fold decrease or 1.4–2.3 fold increase).

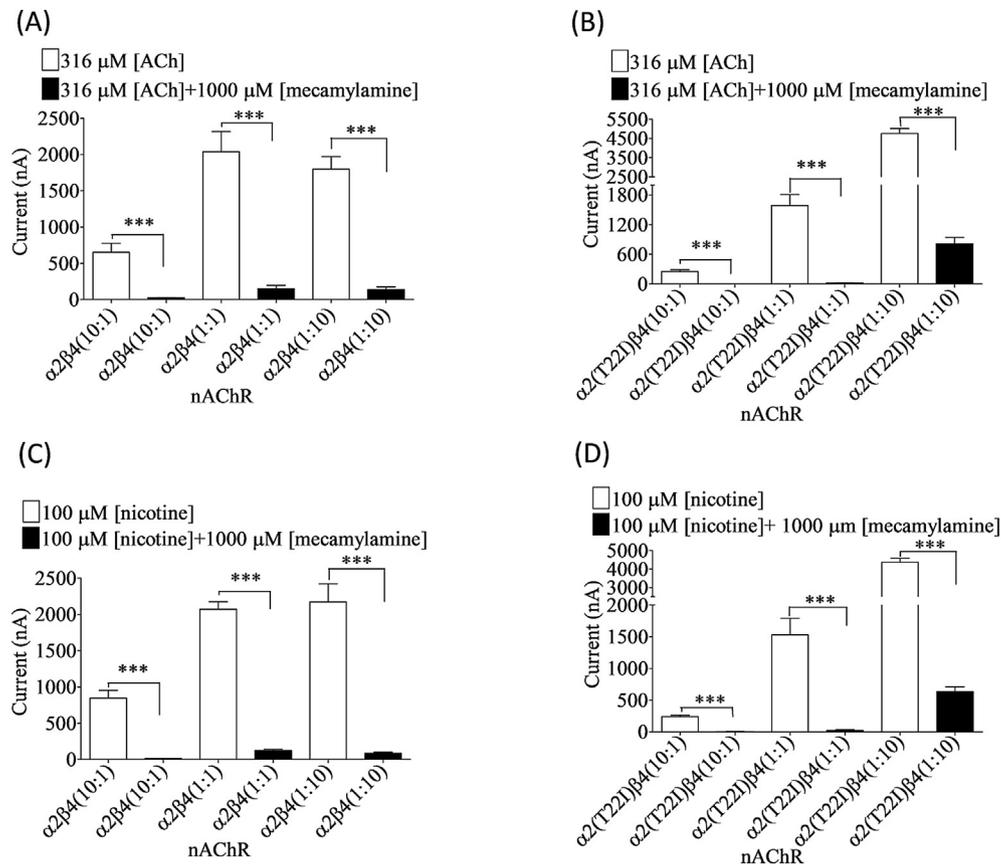
An alternate explanation of the data, which contradicts the above interpretation, appears to favorably explain the 1.4–4-fold decrease in agonist potencies at some  $\alpha 2\beta 2$  or  $\alpha 2\beta 4$  isoforms. An increased representation of SP mutant  $\alpha 2$  subunits in functional  $\alpha 2^*$ -nAChR complexes likely to be the reason for reduction in agonist sensitivities. Thus, the T221 mutation in the SP of the nAChR  $\alpha 2$  subunit is to favor the expression of LS rather than HS isoforms of both  $\alpha 2\beta 2$ - and  $\alpha 2\beta 4$ -nAChR under circumstances likely to be encountered naturally, i.e., when subunit ratios are comparable (1:1). The mutation similarly pushes both LS (10:1) and HS (1:10)

isoforms of  $\alpha 2\beta 2$ -nAChR to lower sensitivity states. That being said, the mutation is largely ineffective in altering agonist potency for LS  $\alpha 2\beta 4$ -nAChR formed at 10:1  $\alpha 2$ : $\beta 4$  subunit ratios. However, this can be explained if those receptors already are predominantly of the LS ( $\alpha 2$ ) $_3(\beta 4)_2$ -nAChR nature.

There could be differences in how WT and mutant subunits are processed in model systems such as oocytes, human cell lines, or naturally in neurons or other cell types. These differences could contribute to and explain some of the differences in properties of wild-type or SP mutant  $\alpha 2$ -nAChRs (Krashia et al., 2010; Sivillotti et al., 1997). Moreover, the facts that several nAChR subtypes exist as isoforms with different subunit stoichiometries and agonist sensitivities and efficacies also offers a mechanism for manifestation of SP mutation effects.

## 5. Conclusion

In summary, we have shown that  $\alpha 2(T221)$  mutation in the SP of human nAChR  $\alpha 2$  subunit gives rise to changes in the function of  $\alpha 2^*$ -nAChR. Despite the mutation being in a region that would be expected to be cleaved off in the mature protein its effect could be measured in oocyte expression system using TEVC recordings. Perhaps the simplest, viable interpretation of the data is that the ultimate effect of the mutation is to favor, generally, formation of low sensitivity  $\alpha 2^*$ -nAChR. We hypothesize that lower sensitivity of human  $\alpha 2^*$ -nAChR to nicotine could contribute to increased



**Fig. 6.** Mecamylamine inhibits agonist induced peak current responses of WT or mutant  $\alpha 2\beta 4$ -nAChRs. Current responses of oocytes injected with (A) 10:1 ( $n = 4$ ), 1:1 ( $n = 4$ ), or 1:10 ( $n = 5$ ) ratio of  $\alpha 2:\beta 4$  subunit cRNAs; and (B) 10:1 ( $n = 3$ ), 1:1 ( $n = 9$ ), or 1:10 ( $n = 4$ ) ratio of  $\alpha 2(T22):\beta 4$  subunit cRNAs and responding to the application of 316  $\mu\text{M}$  ACh was significantly ( $p < 0.001$ ) inhibited by 1000  $\mu\text{M}$  mecamylamine. Similarly, current responses of oocytes injected with (C) 10:1 ( $n = 7$ ), 1:1 ( $n = 11$ ), or 1:10 ( $n = 10$ ) ratio of  $\alpha 2:\beta 4$  subunit cRNAs; and 10:1 ( $n = 5$ ), 1:1 ( $n = 11$ ), or 1:10 ( $n = 4$ ) ratio of  $\alpha 2(T22):\beta 4$  subunit cRNAs and responding to the application of 100  $\mu\text{M}$  nicotine was significantly ( $p < 0.001$ ) inhibited by 1000  $\mu\text{M}$  mecamylamine. Data are presented as the mean  $\pm$  SEM, with numbers of individual oocytes tested ( $n$ ) as indicated. Comparisons between groups were analyzed by Student's  $t$  test (two-tailed; \*\*\*,  $p < 0.001$ ).

susceptibility to ND. We further propose that the nAChR  $\alpha 2$  SP mutation offers a useful model for studying and more generally establishing roles for SP in control of expression and function of members in the cys-loop family of neurotransmitter receptors. Hence SP could be a novel target for manipulating nAChR function and associated diseases.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2014.01.021>.

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