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Two rare variations, D478N and D478E, that occur at the same amino acid residue in nicotinic acetylcholine receptor (nAChR) α2 subunit influence nAChR function

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Abstract

There occur two rare variations, Asp(D)478Asn(N) and Asp(D)478Glu(E), in the putative cytoplasmic amphipathic α-helices of human nicotinic acetylcholine receptor (nAChR) α2 subunit as a result of mutation in the 1st (G→A: rs141072985) and 3rd (C→A: rs56344740) nucleotide of its 478th triplet codon (**G**A**C**). We assessed the effects of these two variations on the function of α2β2- and α2β4-nAChRs as they could alter the electronegativity and/or the structure of the cytoplasmic 'portals' (framed by subunit amphipathic α-helices) necessary for obligate ion permeation from extracellular space to cytoplasm. We injected decreasing ratio of subunit cRNAs (α:β; 10:1, 1:1 and 1:10) into *Xenopus* oocytes to express putative low sensitivity (LS; 10:1), intermediate-sensitivity (IS; 1:1) and high sensitivity (HS; 1:10) isoforms of wild type and variant α2β2- and α2β4-nAChRs. Two-electrode voltage clamp analyses indicate that the agonist (ACh or nicotine) induced peak current responses (I_{max}) of α2β2-nAChR isoforms and those of α2β4-nAChR isoforms are increased (1.3–4.7-fold) as a result of D478E variation. The α2 subunit D478N variation only increases the I_{max} of IS (~2-fold) or HS (1.4–2.1-fold) α2β2-nAChRs. Concentration-response curves constructed indicate no effect on agonist sensitivities of LS and HS isoforms of α2β2- or α2β4-nAChRs as a result of either variation in α2 subunit. Between the two variant nAChRs, α2(D478E)*-nAChR isoforms generally yield higher I_{max} than those of respective α2(D478N)*-nAChR isoforms. These effects could be attributed to alteration in cytoplasmic 'portals' and/or ion permeation through it owing to change in amino acid electronegativity (D→N) and side chain length (D→E) in nAChR α2 subunit.

Keywords

Nicotinic acetylcholine receptor; single nucleotide variation; missense mutation; receptor structure-function; electrophysiology

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1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are the principal binding site for exogenous psychoactive ligand nicotine in the brain. Mammalian neuronal nAChRs are composed of five nAChR subunits and principally formed either as a homomer (e.g., α7-nAChR) or heteromer (e.g., α2β2-, α3β2-, α4β2-, α6β2-, α9α10-nAChR). Heteromeric nAChRs are formed by the various combination of α and β or combination of α subunits alone but homomeric nAChRs are composed of the same α subunits. Each nAChR subunit has a large N-terminal extracellular domain (ECD/E1) that is followed by 4 trans-membrane domains (TM I-IV) and a small C-terminal extracellular domain (CTD). TM I and TM II; TMII and TM III; and TM III and TM IV are connected to each other via a small cytoplasmic loop (C1), a small extracellular loop (E2), and a large cytoplasmic loop (C2 or cyto-loop) respectively (Lukas et al., 1999).

The structure of substantial portion of the cyto-loop is largely unknown. A region located adjacent to the TM IV in the cyto-loop is dubbed as membrane-associated (MA or HA) stretch (Finer-Moore and Stroud, 1984) and is resolved as an α-helical structure in the crystal structure of *Torpedo* muscle nAChR (Unwin, 2005). Other studies predict it to be mostly unfolded and having α-helical structures within two segments of the cyto-loop (Kukhtina et al., 2006). These cytoplasmic amphipathic α-helices from the five participating subunits of a given nAChR in conjunction with the loop that connects TM I to TM II is thought constitute an intracellular vestibule perforated by five narrow fenestrations (‘portals’) that serve as obligate pathways through which ion must flux after emerging from the ion channel gate formed by TM II α-helices (Miyazawa et al., 2003; Unwin, 2005). Several studies also prove that cyto-loop of the participating subunits influence single channel conductance (γ) and ion selectivity of the nAChRs and other members of the cys-loop family of receptors (Carland et al., 2013; Hales et al., 2006; Kelley et al., 2003; Thompson and Lummis, 2003).

Mutations in and around the MA stretch region has been shown to influence the function of nAChRs. A single nucleotide polymorphism (SNP: rs16969968, D398N) that leads to the substitution of an aspartate (D) for an asparagine (N) in the human nAChR α5 subunit, to date the most strongly associated SNP in several association studies of nicotine dependence (ND) and other correlated traits (Thorgeirsson et al., 2008), possibly belongs to the MA stretch region. *In vitro* functional studies have demonstrated that nAChRs harboring the amino acid encoded by the minor allele (N398), which is the risk allele, show reduced (Bierut et al., 2008; George et al., 2012; Kuryatov et al., 2011) or unaltered (Li et al., 2011) response to the nicotinic agonists. Human carriers of the minor allele (N398) show decreased intrinsic resting connectivity strength in the dorsal anterior cingulate-ventral striatum/extended amygdala circuit (Hong et al., 2010). Also a similarly located mutation (S435R) in the nAChR β4 subunit abolishes β4 subunit specific activity of α3β4-nAChRs (Frahm et al., 2011).

There occur two rare variations (rs141072985: D478N and rs563447740: D478E; Fig. 1) in the human (h) nAChR α2 subunit. These are putatively located in its cytoplasmic amphipathic α-helices (MA stretch). Occurrence of the D478N variation in nAChR hα2

subunit is as a result of a rare missense mutation in the 1432nd nucleotide (i.e., the 1st nucleotide of the 478th triplet codon; **G**AC→**A**AC: underlining indicates nucleotide change due to mutation) of coding sequence (CDS) of the nAChR hα2 mRNA (NCBI Reference Sequence: NM_000742.3, Entrez Gene ID: 1135). Also the occurrence of the D478E variation in nAChR hα2 subunit is as a result of another rare missense mutation in the same 478th triplet codon of the nAChR hα2 mRNA but at the 3rd nucleotide of the triplet codon (GA**C**→GA**A**). Current data indicate that the E478 allele is overrepresented (minor allele frequency; MAF=0.0023) than N478 allele (MAF=0.0009) and both minor alleles are relatively more prevalent in European American populations than in African American populations (source: dbSNP, NCBI and NHLBI Grand Opportunity Exome Sequencing Project).

In this study we assess the effects on human α2*-nAChR ('*' indicates presence of additional subunits) function of these two rare variations (D478N and D478E) in nAChR hα2 subunit. We injected into *Xenopus* oocytes decreasing ratio of α:β subunit cRNAs (10:1, 1:1 and 1:10) to express putative low-sensitivity (LS; 10:1), intermediate-sensitivity (IS; 1:1) and high sensitivity (HS; 1:10) isoforms of wild type (WT) α2β2- and α2β4-nAChRs as well as those of variant α2(D478N)β2-, α2(D478E)β2-, α2(D478N)β4- and α2(D478E)β4-nAChRs. Our results indicate that these two variations principally affect the agonist induced peak current responses of both α2β2- and α2β4-nAChRs to various degrees. These results also demonstrate that amino acid (AA) variations that could alter the electronegativity and/or structure of the cytoplasmic portals framed by amphipathic α-helices could affect the function of nAChRs.

2. Experimental procedures

2.1 Bioinformatics analyses

Amino acid sequences of human nAChR alpha (α1-7, α9-10) subunits and nAChR α2 subunits from various organisms are aligned, separately, using ClustalW (Fig. 1). Changes that might occur in the secondary or 3-D structure of the nAChR α2 protein due to the presence of an asparagine (N) or glutamate (E) instead of an aspartate (D) at 478th residue was assessed using several web based protein prediction programs such as HHpred (Homology detection and structure prediction by HMM-HMM comparison) (http://toolkit.tuebingen.mpg.de/hhpred), 3D-Jigsaw (http://bmm.cancerresearchuk.org/~3djigsaw/), PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/); etc. A homology model of the nAChR hα2 subunit retrieved using 3-D-Jigsaw protein server was rendered using UCSF Chimera, a program for interactive visualization and analysis of molecular structures (http://www.cgl.ucsf.edu/chimera/) (Fig. 1). Whether the change in nucleotide as a result of the mutation 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 nAChR hα2 mRNA in miRBASE (www.mirbase.org), TargetScan (www.targetscan.org), and snoRNA-LBME-db (www-snorna.biotoul.fr) registries.

2.1 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 made daily in oocyte Ringer's solution (OR2: 92.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES; pH 7.5.) from frozen stocks and were diluted as needed.

2.2 Subcloning and mutagenesis of nicotinic receptor subunits

Human nAChR α2, β2 and β4 subunits were subcloned into the oocyte expression vector pGEMHE previously (Dash et al., 2012). A synthetic, nAChR hβ2 subunit with nucleotide sequences optimized for better heterologous expression ($h\beta2_{opt}$) was made (Invitrogen/GENEART, Burlingame, CA) and subcloned into the pCI vector (Promega, San Luis Obispo, CA) previously (Dash et al., 2011a; Dash and Lukas, 2012; Dash et al., 2014). Variations in the nAChR hα2 subunit were introduced in the pGEMHE background using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotides used for mutating aspartate (D) to asparagine (N) at AA residue 478 in the nAChR hα2 subunit are 5′-gtgcactacattgcc**A**accacctgcggtctg-3′ (D478N-Forward, capitalization indicates the nucleotide changed from the WT) and 5′-cagaccgcaggtggtTggcaatgtagtgcac-3′ (D478N-Reverse). Similarly oligonucleotides used for mutating aspartate (D) to glutamate (E) at AA residue 478 in the hα2-nAChR subunit are 5′-cactacattgccga**A**cacctgcggtctgag-3′ (D478E-Forward) and 5′-ctcagaccgcaggtgTtcggcaatgtagtg-3′ (D478E-Reverse). Identities and accuracies of all WT or mutant subunit cDNAs were confirmed by sequencing and comparing them to the nucleotide/protein reference sequences available in GenBank.

2.3 Preparation and dilution of cRNAs for α2*-nAChR expression studies

All pGEMHE plasmids were linearized immediately downstream of the 3′-polyadenylation sequence. *Nhe*I was used to linearize nAChR hα2, hα2(D478N), hα2(D478E) and hβ4 subunit-containing plasmids, and *SwaI* was used to linearize $h\beta2_{opt}$ subunit containing plasmids. Full length capped mRNA (i.e., cRNA) was transcribed from multiple batches of linearized plasmids using mMESSAGE mMACHINE® T7 Kit (Invitrogen/Ambion Inc., CA, USA) and following manufacturer's instructions. Integrity and quality of the cRNA was checked by electrophoresis and UV-spectroscopy.

To express WT or variant hα2*-nAChRs in oocytes, we planned to inject cRNA corresponding to each α or β subunit in ratios of 10:1, 1:1 or 1:10. Although there is no guarantee that 1:1 approach will result in expression of equal amounts of each subunit protein, it is more likely to do so than an approach where biased ratios (10:1 or 1:10) of subunit-encoding cRNAs are injected into oocytes. The latter kind of approach has been proven to favor formation of low or high sensitivity (LS- or HS-) receptors, respectively, to nicotinic agonists (Dash et al., 2014; Nelson et al., 2003; Zwart and Vijverberg, 1998). Injection of cRNAs mixed in ratio of 10:1 α:β subunit would bias toward predominant expression of putative LS [i.e., $(\alpha2)_3(\beta2)_2$-, $\{\alpha2(D478N)\}_3(\beta2)_2$-, $\{\alpha2(D478E)\}_3(\beta2)_2$-, $(\alpha2)_3(\beta4)_2$-, $\{\alpha2(D478N)\}_3(\beta4)_2$- or $\{\alpha2(D478E)\}_3(\beta4)_2$-] nAChRs. Injection of cRNAs mixed in ratio of 1:10 α:β subunit would bias toward predominant expression of putative HS

[i.e., $(\alpha 2)_2(\beta 2)_3$-, $\{\alpha 2(D478N)\}_2(\beta 2)_3$-, $\{\alpha 2(D478E)\}_2(\beta 2)_3$-, $(\alpha 2)_2(\beta 4)_3$-, $\{\alpha 2(D478N)\}_2(\beta 4)_3$-, or $\{\alpha 2(D478E)\}_2(\beta 4)_3$-] nAChRs. nAChRs expressed as a result of cRNA injection in ratio of 1:1 α:β subunit would attain intermediate sensitivity (IS) relative to those of LS and HS nAChR isoforms as 1:1 ratio of α:β subunit cRNA injection would lead to a mixed population of LS- and HS-nAChRs (Dash et al., 2014).

Concentration of cRNAs for each nAChR α and β subunit, WT or mutant, was initially adjusted to 777 ng/μL. cRNAs for each α and β subunit were further diluted 10 fold (i.e., final concentration of 77.7 ng/μL) for use in biased ratio (α:β::10:1 or 1:10) expression studies. From each batch of synthesized cRNAs several cRNA mixtures corresponding to 10:1, 1:1 and 1:10 α:β subunit ratio were prepared (by mixing equal volume of cRNA for each α and β subunit) and stored at −80°C until further use. Injection of 69 nL of cRNA mixtures into each oocyte would deliver: ~27 ng of cRNAs for each α and β subunit for 1:1 ratio expression studies; ~27 ng and ~2.7 ng of cRNAs respectively for each α and β subunit for 10:1 ratio expression studies; or ~2.7 ng and ~27 ng of cRNAs respectively for each α and β subunit for 1:10 ratio expression studies.

2.4 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, Charlottesville, VA, USA. Matured female frogs (*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). The 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 $MgCl_2$, 1 $CaCl_2$, 1 Na_2HPO_4, 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 the 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 microliter (4 μL) cRNA mixture was drawn up into the micropipette and 69 μL of cRNA was injected into each oocyte using a Nanoject II microinjection system (Drummond Scientific, Broomall, PA).

2.5 Oocyte electrophysiology

One to 5 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 pClamp10.2. Electrodes contained 3 M KCl and had a resistance of 1–2 MΩ. Drugs (agonists and antagonists) were prepared daily in OR2 and 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 acetylcholine receptor (mAChR) responses. Drug applications lasted for at least 5 seconds. All current measurements were conducted or checked in at least two batches of oocytes.

Earlier studies indicated coexpression of a codon optimized human nAChR β2 subunit ($h\beta2_{opt}$) enhances the functional responsiveness of human β2*-nAChRs expressed in oocytes (Dash et al., 2014). Advantages of use of such codon-optimized nAChR subunits were also demonstrated previously (Dash et al., 2011b; Dash and Lukas, 2012; Slimko and Lester, 2003). Hence all results reported for β2*-nAChRs are as a result of coexpression of nAChR $h\beta2_{opt}$ subunit together with other nAChR subunits.

2.6 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 nAChRs. Current responses to 100 μM nicotine or 100 μM ACh in the presence of 1 μM atropine were less than 5–10 nA (data not shown).

2.7 Data analyses

Raw data was collected from at least 3 individual oocytes (n=3) 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). Generation of true I_{max} values (maximal current responses) and EC_{50} (concentration for half-maximal activation) values for different nAChR subunit combinations required assessment based on complete concentration-response (CR) relationships. Concentration-response curves were constructed from the average values of the normalized current responses of individual oocytes by fitting them to Hill equation (Prism 4, sigmoidal dose-response with variable slope, bottom constant equal to zero and top constant equal to 1; GraphPad Software, San Diego, CA). For an individual oocyte current responses to a series of agonist concentrations are normalized to the maximal response (I_{max}) of that agonist.

EC_{50} values for variant α2-nAChR isoforms were compared to those of the WT α2-nAChR isoforms expressed using comparable cRNA ratios. Also EC_{50} values for the variant α2(D478E)*-nAChR isoforms were compared to those of the variant α2(D478N)*-nAChR isoforms expressed using comparable cRNA ratios. Also the effect of the cRNA ratio variations (α:β 10:1 or 1:10) on the EC_{50} values of WT and variant α2*-nAChRs were 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. Also 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 levels of functional nAChR expression, 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 expression as described previously (Dash et al., 2011a, 2012; Dash et al., 2011b; Dash and Lukas, 2012; Groot-Kormelink et al., 2001). 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.

I_{max} values in response to most efficacious concentration of ACh or nicotine were compared across different subunit combinations. The I_{max} value of the variant α2*-nAChRs was compared (one-way ANOVA with Tukey's post hoc comparison: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$) to that of the WT α2-nAChRs expressed using comparable cRNA ratios. Also the I_{max} value of the variant α2(D478E)*-nAChRs was compared to that of α2(D478N)*-nAChRs expressed similarly (one-way ANOVA with Tukey's post hoc comparison: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$). The results of the study also warranted comparisons of (student's t-test: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$) I_{max} values between LS and HS isoforms of WT or variant α2-nAChRs.

3. Results

3.1 In silico analysis indicated possible functional consequences for D478N or D478E variation in nAChR α2 subunit

The degree of evolutionary conservation of an aspartate (D), asparagine (N) or glutamate (E) residue located at the 478th position in the nAChR α2 subunit was explored among human nAChR α subunits and nAChR α2 subunits from several other organisms (Fig. 1). Multiple protein sequence alignment results indicate that any of these three AA residues (D, N or E) located at the 478th position of nAChR hα2 subunit is not conserved in other human nAChR α subunits. However, nAChR α2 subunit protein sequences aligned from other organisms indicate the presence of a conserved aspartate (D) residue at this position (Fig. 1B). These results also indicate the presence of a charged residue (Asp, Glu, Lys or Arg) more often than an uncharged residue (Asn) at this position. Hence introduction of a negatively charged residue with a longer side chain (E) or an uncharged residue (N) in lieu of a negatively charged residue (D) in a fairly conserved region of the human nAChR α2 subunit is expected to have functional consequences.

Results from web-based secondary (2-D) or tertiary (3-D) structure prediction web servers indicate that the 478th AA residue of nAChR α2 subunit (i.e., D478, N478 or E478) could be part of an alpha helix (MA stretch) that typically precedes the TM IV of a typical nAChR subunit [Fig 1(C)] and participate in the constitution of the so called intracellular vestibule/cytoplasmic portals responsible for obligate ion permeation into the cytoplasm. In this

region the 398th residue of nAChR hα5 subunit (i.e., D398), equivalent to the 471st residue of nAChR hα2 subunit (i.e., E471) (Fig. 1; arrow mark), has drawn great attention for its role in ND and other diseases (Frahm et al., 2011; George et al., 2012; Hong et al., 2010; Kuryatov et al., 2011; Li et al., 2011; Tammimaki et al., 2012; Thorgeirsson et al., 2008). The N398 residue (i.e., the mutant/risk allele) of nAChR α5 subunit is predicted to alter the structure of intracellular vestibule/cytoplasmic portals of α3β4α5-nAChR (Frahm et al., 2011). Based on these findings we anticipate that the D478N or D478E variation in nAChR α2 subunit likely to affect the structure and function of α2*-nAChRs.

Database (noted above) scanning did not indicate introduction or destruction of any miRNA and/or other (snoRNAs and scaRNAs) RNA regulatory sites in the mRNA sequence of nAChR α2 subunit as a result of either rs141072985 or rs56344740 rare variation in it.

3.2 α2β2-nAChR function is altered as a result of D478N or D478E variation in nAChR α2 subunit

nAChRs were expressed by injecting mixtures of cRNAs for α2 plus β2 subunits, α2(D478N) plus β2 subunits or α2(D478E) plus β2 subunits in equal quantities (i.e., 1:1, presumably forming a mixture of receptor isoforms), 10:1 ratio [presumably favoring formation of LS: $(\alpha2)_3(\beta2)_2$, $\{\alpha2(D478N)\}_3(\beta2)_2$ or $\{\alpha2(D478E)\}_3(\beta2)_2$-nAChR], or 1:10 ratio [presumably favoring formation of HS: $(\alpha2)_2(\beta2)_3$, $\{\alpha2(D478N)\}_2(\beta2)_3$ or $\{\alpha2(D478E)\}_2(\beta2)_3$-nAChR]. We generated the CR curves for both the presumed LS and HS isoforms and measured the I_{max} values for all the three isoforms of α2β2-nAChRs.

3.2.1 Agonist sensitivities of LS or HS α2β2-nAChR isoforms are largely unaffected by D478N or D478E variation in nAChR α2 subunit—

As expected α2β2-, α2(D478N)β2- and α2(D478E)β2-nAChRs expressed using α and β subunit cRNAs mixed in a ratio of 1:10 displayed highest agonist sensitivity (HS-nAChR; ACh EC_{50}: 1.4, 2.2 and 2.4 μM respectively; and nicotine EC_{50}: 0.83, 1.3 and 1.5 μM respectively); and those expressed using 10:1 ratio of α:β subunit cRNAs were least sensitive to agonists (LS-nAChR; ACh EC_{50}: 26, 23 and 27 μM; and nicotine EC_{50}: 6.3, 7.4 and 6.8 μM respectively) (Figs. 2 and S1, Table 1). Comparisons between isoforms (i.e., relationship between LS and HS-nAChRs) indicated lower ($p < 0.05$) potencies for ACh or nicotine acting at LS α2β2-, α2(D478N)β2- and α2(D478E)β2-nAChRs than acting at respective HS receptors. Hence as expected an increase in receptor sensitivity to ACh or nicotine is observed as the [α2, α2(D478N) or α2(D478E)]:β2 subunit message ratio is decreased.

Further comparative analyses indicated that ACh or nicotine EC_{50} values of LS or HS α2β2-nAChRs were not affected by either D478N or D478E variation in α2 subunit.

3.2.2 Peak current response of α2β2-nAChR isoforms are increased as a result of D478N or D478E variation in nAChR α2 subunit—

I_{max} is mostly decreased for HS α2β2- (~31% for ACh; and ~73% for nicotine, $p < 0.001$), HS α2(D478N)β2- (~43% for nicotine, $p < 0.05$) or HS α2(D478E)β2- (~52% for ACh, $p < 0.01$; and ~78% for nicotine, $p < 0.001$) nAChRs compared to those of their respective LS counterparts (Fig. 3; Table 1).

Further comparative analyses further indicated that I_{max} for HS (~40%/ACh, $p < 0.01$; and ~2.1 fold/nicotine, $p < 0.001$) or IS (~80%/ACh, $p<0.01$; and ~2.2 fold/ nicotine, $p < 0.001$) α2β2-nAChRs is increased as a result of D478N variation in nAChR α2 subunit. Also, I_{max} of LS (~2.4 fold/ACh, $p < 0.01$; and ~3 fold/ nicotine, $p < 0.001$), IS (~2.4 fold/ ACh, $p<0.01$; and ~4.7 fold/nicotine, $p < 0.001$) or HS (~60%/ACh, $p<0.01$; and ~2.3 fold/ nicotine, $p < 0.001$) α2β2-nAChRs is increased as a result of D478E variation in nAChR α2 subunit.

3.3 α2β4-nAChR function is altered as a result of D478E, but not D478N, variation in nAChR α2 subunit

In order to determine whether the α2 subunit variations (D478N or D478E) would have similar effects on the function of α2β4-nAChRs, studies were also conducted by injecting cRNAs for α2 and β4 subunits, α2(D478N) and β4 subunits or α2(D478E) and β4 subunits in equal quantities (i.e., 1:1, presumably forming a mixture of receptor isoforms or IS isoforms), at a 10:1 ratio [presumably favoring formation of LS: $(\alpha2)_3(\beta4)_2$, $\{\alpha2(D478N)\}_3(\beta4)_2$ or $\{\alpha2(D478E)\}_3(\beta4)_2$-nAChRs], or at a 1:10 ratio [presumably favoring formation of HS: $(\alpha2)_2(\beta4)_3$, $\{\alpha2(D478N)\}_2(\beta4)_3$ or $\{\alpha2(D478E)\}_2(\beta4)_3$-nAChRs]. All of these nAChRs were functionally activated by ACh or nicotine (Figs. 4, 5 and S1; Table 1). We generated the CR curves for both the presumed LS and HS isoforms and measured the I_{max} values for all the three isoforms of α2β4-nAChRs.

3.3.1 Agonist sensitivities of LS or HS α2β4-nAChR isoforms are not affected by D478N or D478E variation in nAChR α2 subunit—EC_{50} values for ACh were 40 and 10 μM for LS and HS α2β4-nAChRs, respectively ($p < 0.001$ for HS compared to LS); 45 and 13 μM for LS and HS α2(D478N)β4-nAChRs, respectively ($p < 0.001$ for HS compared to LS) and 35 and 13 μM for LS and HS α2(D478E)β4-nAChRs, respectively ($p < 0.001$ for HS compared to LS (Fig. S1; Table 1). As for nicotine EC_{50} values were 12 and 4 μM for LS and HS α2β4-nAChRs, respectively ($p < 0.001$ for HS compared to LS); 11 and 4.6 μM for LS and HS α2(D478N)β4-nAChRs, respectively ($p < 0.01$ for HS compared to LS); and 10 and 4.5 μM for LS and HS α2(D478E)β4-nAChRs, respectively ($p < 0.01$ for HS compared to LS) (Fig. S1; Table 1). As expected sensitivity to ACh or nicotine is increased as the ratio of [α2, α2(D478N) or α2(D478E)]:β4 subunit message is decreased. Further comparative analyses indicated that ACh or nicotine sensitivity of LS or HS α2β4-nAChRs was not affected by either D478N or D478E variation in α2 subunit.

3.3.2 Peak current response of α2β4-nAChR isoforms are increased as a result of D478E, but not D478N, variation in nAChR α2 subunit—Comparison of I_{max} between isoforms (i.e., relationship between LS- and HS-nAChRs; Fig. 3; Table 1) indicated an increase in I_{max} for HS α2β4- (2.2-fold for ACh, $p < 0.001$; and 1.7-fold for nicotine, $p < 0.05$), HS α2(D478N)β4- (2.9-fold for ACh, $p < 0.001$; and 2.1-fold for nicotine, $p < 0.001$) or HS α2(D478E)β4- (1.6-fold for ACh, $p < 0.001$; and 1.6-fold for nicotine, $p < 0.01$) nAChRs relative to those of their respective LS counterparts (Fig. 3; Table 1).

Comparative analyses indicated that I_{max} of LS, IS or HS α2β4-nAChRs are not affected as a result of D478N variation in nAChR α2 subunit. However, I_{max} of LS (70%/ACh; 50%/nicotine; $p < 0.05$), IS (50%/ACh; 30%/nicotine; $p < 0.05$) or HS (30%/ACh; 50%/nicotine; $p < 0.05$) α2β4-nAChRs are increased as a result of D478E variation in nAChR α2 subunit.

3.4 Peak current responses of α2(D478N)(β2 or β4)-nAChR isoforms are generally lower than those of respective α2(D478E)(β2 or β4)-nAChR isoforms

The agonist (ACh or nicotine) EC_{50} values for the LS and HS isoforms of α2(D478N)(β2 or β4)-nAChRs did not differ ($p > 0.05$) from those of their respective isoforms of α2(D478E)(β2 or β4)-nAChRs.

Agonist elicited peak current responses of all the 3 (LS, IS and HS) isoforms of α2(D478E)β2-nAChRs are generally higher than those of their respective isoforms of α2(D478N)β2-nAChRs but the I_{max} for LS (~2.5 fold/ACh, $p < 0.01$; and ~3 fold/nicotine, $p < 0.01$) and IS (~2.1 fold/nicotine, $p < 0.01$) α2(D478E)β2-nAChRs significantly exceeded those of their respective isoforms of α2(D478N)β2-nAChRs. Similarly I_{max} of α2(D478E)β4-nAChR isoforms are generally higher than those of their respective α2(D478N)β4-nAChR isoforms but the agonist elicited I_{max} for LS (~2.2 fold/ACh, $p < 0.01$; and ~1.5 fold/nicotine, $p < 0.01$) α2(D478E)β4-nAChRs significantly exceeded those of LS α2(D478N)β4-nAChRs.

3.5 Mecamylamine inhibits peak current responses of wild type and variant α2(β2 or β4)-nAChRs

Mecamylamine blocked 94% (2071 nA vs. 122 nA; while the former number is the mean peak current induced by 100 μM nicotine alone, the latter one is the mean peak current in the additional presence of 1000 μM mecamylamine), 94% (2615 nA vs. 169 nA) and 90% (2862 nA vs. 283 nA) of the nicotine induced peak current responses of IS isoforms of α2β4-, α2(D478N)β4- and α2(D478E)β4- nAChRs respectively. Also 98 % (1211 nA vs. 22 nA), 99% (1173 nA vs. 14 nA) and 98% (1508 nA vs. 29 nA) of nicotine induced peak current responses of LS isoforms of α2β4-, α2(D478E)β4- and α2(D478N)β4-nAChRs were blocked by mecamylamine respectively. Nicotine induced peak current responses of HS isoforms of α2β4-, α2(D478N)β4- and α2(D478E)β4- nAChRs were inhibited 96% (2114 nA vs. 86 nA), 95% (2717 nA vs. 142 nA) and 97% (2471 nA vs. 79 nA) by mecamylamine respectively. Similarly mecamylamine at 1000 μM concentration inhibited agonist (316 μM ACh) induced peak current responses of LS, IS and HS isoforms of α2β4-, α2(D478N)β4- or α2(D478E)β4-nAChRs by 92 to 99 % (data not shown).

Mecamylamine at 1000 μM concentration completely abolished the ACh or nicotine induced I_{max} responses of LS, IS and HS isoforms of α2β2-, α2(D478N)β2- or α2(D478E)β2-nAChR (data not shown).

4. Discussion

In this study we wanted to know whether two rare variations (D478N and D478E) that occur in the same AA residue in the amphipathic α-helices of human nAChR α2 subunit would affect the function of human α2β2- and α2β4-nAChRs. The principal conclusions of the

study are as follows. The D478E variation in nAChR α2 subunit increases the ACh or nicotine induced I_{max} of α2β2- and α2β4-nAChRs whether they are expressed as LS, IS or HS receptors. However, the D478N variation increases the ACh or nicotine induced I_{max} of α2β2-nAChRs expressed as IS or HS isoforms. Between the two variant receptors; LS, IS and HS isoforms of α2(D478E)*-nAChRs yield generally higher agonist induced I_{max} than those of respective isoforms of α2(D478N)*-nAChRs. ACh or nicotine sensitivity of α2β2 or α2β4-nAChRs expressed as LS or HS isoforms are not altered as a result of either variation in nAChR α2 subunit. Hence, it appears that the function of both α2β2- and α2β4-nAChRs is influenced by D478E variation in nAChR α2 subunit but the function of α2β2-nAChR is additionally influenced by D478N variation in nAChR α2 subunit. All forms (LS, IS and HS) of WT or variant α2(β2 or β4)-nAChRs are inhibited by mecamylamine. These results indicate that the amphipathic helix variations in nAChR α2 subunit influence the function (and potentially in the structure) human α2*-nAChRs.

Cytoplasmic vestibules formed by the amphipathic helices of participating nAChR subunits serve as central conduits for ion permeation to the cytoplasm after their emergence from the channel gate. These vestibules like TM II and some residues in the ECD determine the single channel conductance and ion selectivity of nAChRs and other members of the cys-loop family of receptors (Hales et al., 2006; Kelley et al., 2003; Thompson and Lummis, 2003). Cytoplasmic vestibule presents five parallel electronegative conduction pathways, rather than a single, central conduit (Unwin, 2005). Changes in AA residues that line the ion conduction pathways and/or those of α-helices that contribute to formation of the conducting pathways influence function of nAChRs (Frahm et al., 2011). The D398N variation in nAChR α5 subunit commonly associated with ND and other diseases is mapped to the cytoplasmic vestibular regions (i.e. MA stretch) and is shown to modulate function of nAChRs both *in vitro* and *in vivo* (Frahm et al., 2011; George et al., 2012; Kuryatov et al., 2011; Tammimaki et al., 2012). In these studies it is shown that D398N variation in nAChR α5 subunit decreases the agonist response of α4β2- or α3β4-nAChRs (George et al., 2012; Kuryatov et al., 2011) or doesn't affect the pharmacology of α3β4-nAChR activation (Li et al., 2011). Also the S435R mutation in the nAChR β4 subunit, which belongs to the MA stretch, abolishes β4 specific activity of α3β4-nAChRs (Frahm et al., 2011). Based on these findings we anticipated that D478N and/or D478E variation in the MA stretch of nAChR α2 subunit would have functional consequences and our results precisely indicate that.

Our results indicate that substitution of a glutamate (E) for an aspartate (D) residue at the 478^{th} residue of the nAChR α2 subunit influence the peak current responses of α2β2- and α2β4-nAChRs. This is most likely due to structural changes brought in the obligatory ion conduction pathway/ cytoplasmic vestibules as overall electronegativity (both D and E are negatively charged) of the conduction pathways won't be affected by this mutation. Structural changes in the cytoplasmic vestibules could be imposed by the relatively longer side chain of the glutamate (E) than the aspartate (D) residue. Also it could be envisioned that the incumbent changes in pharmacological properties are as a result of the interaction of the variant glutamate (E) residue in one or multiple nAChR α2 subunit(s) with residues of partnering (β2 or β4) or distant (α2, β2 and/or β4) subunits giving rise to isoform specific effects for α2β2- and α2β4-nAChRs.

The substitution of an asparagine (N) for an aspartate (D) at the 478th residue of nAChR α2 subunit has isoform (LS vs. IS vs. HS) and subtype (α2β2- vs. α2β4-nAChR) specific effects. This variation in nAChR α2 subunit does not influence the peak current responses of LS, IS and HS isoforms of α2β4-nAChRs but it increases the peak current responses of IS and HS, but not that of LS, α2β2-nAChRs. These findings are somewhat counterintuitive as the charge neutral asparagine (N) is expected to affect the electronegative environment of the cytoplasmic fenestrations that may hinder the outflow of the cations to the cytoplasm after their emergence from the TM II channel gate. Nonetheless the lack of effect of D478N variation in nAChR α2 subunit on the peak current responses of LS, IS and HS α2β4-nAChRs and LS α2β2-nAChRs is in agreement with the central findings of Li et al. (2011) about the lack of an effect of D398N variation in nAChR α5 subunit on the peak current responses of α3β4α5-nAChRs. There is no precedent in the literature for increase in peak current responses of nAChRs as a result of D to N mutation in HA stretch though similarly located mutation (D398N) in nAChR hα5 subunit either do not affect or reduce the peak current responses of α3β4α5- or α4β2α5-nAChRs (George et al., 2012; Kuryatov et al., 2011; Li et al., 2011). Our results for increase in peak current responses of IS and HS isoforms of α2β2-nAChRs as a result of D478N variation in nAChR α2 subunit add another dimension to the observed effect of an asparagine (N) residue in the amphipathic α-helix and could only be explained as a isoform specific effect at this point.

It is observed that the agonist induced peak current responses α2(D478E)*-nAChR isoforms generally and in some instances significantly ($p < 0.05$) exceed those of respective isoforms of α2(D478N)*-nAChRs. This could be explained by the fact that substitution of a glutamate (E) for an asparagine (N) (i.e., N478E) in nAChR α2 subunit increases the electronegativity of the cytoplasmic fenestrations that may increase the outflow of the cations to the cytoplasm from extracellular space.

The D478N or D478E variation in nAChR α2 subunit does not alter the agonist sensitivities of LS and HS isoforms of the α2β2-nAChRs or those of the α2β4-nAChRs. The general lack of an effect of these two variations, D478N and D478E, in nAChR α2 subunit on agonist sensitivities of the α2β2- and α2β4-nAChR isoforms could be due to their location away from the ligand binding and signal transduction domains.

We conclude that D478E variation in nAChR α2 subunit increases the peak current responses of both α2β2- and α2β4-nAChRs; but the D478N variation in nAChR α2 subunit only increases the peak current responses of α2β2-nAChRs. The increase in peak current responses of α2β2-nAChRs is more pronounced due to D478E variation than the D478N variation in nAChR α2 subunit. These results altogether reinforces the emerging observations that α-helices in the MA (or HA) stretch play an important role in the functional pharmacology of nAChRs and other members of the cys-loop family of receptors. Whether these rare variations play any role in nicotine dependence or other α2*-nAChR associated diseases is not known yet. We won't know such results until new statistical (e.g., rare variation association analysis) and/or biotechnological (e.g., genome editing) tools become available or currently available tools (e.g., Crisper-CAS system) become mainstream.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

ACh	acetylcholine
nAChRs	nicotinic acetylcholine receptors
I_{max}	peak current response
SNP	single nucleotide polymorphism
SNV	Single nucleotide variation
LS	low sensitivity
IS	intermediate sensitivity
HS	high sensitivity
CR	concentration-response
h	human
AA	amino acid
ND	nicotine dependence

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Highlights

- Variations D478N and D478E occur at the same AA residue in human nAChR α2 subunit
- D478E variation increases the current responses (I_{max}) of α2β2- and α2β4-nAChRs
- D478N variation only increases the I_{max} of α2β2 nAChRs
- I_{max} of α2(D478E)*-nAChRs are generally higher than those of α2(D478N)*-nAChRs
- Agonist sensitivity of α2*-nAChRs are largely unaffected by either variation

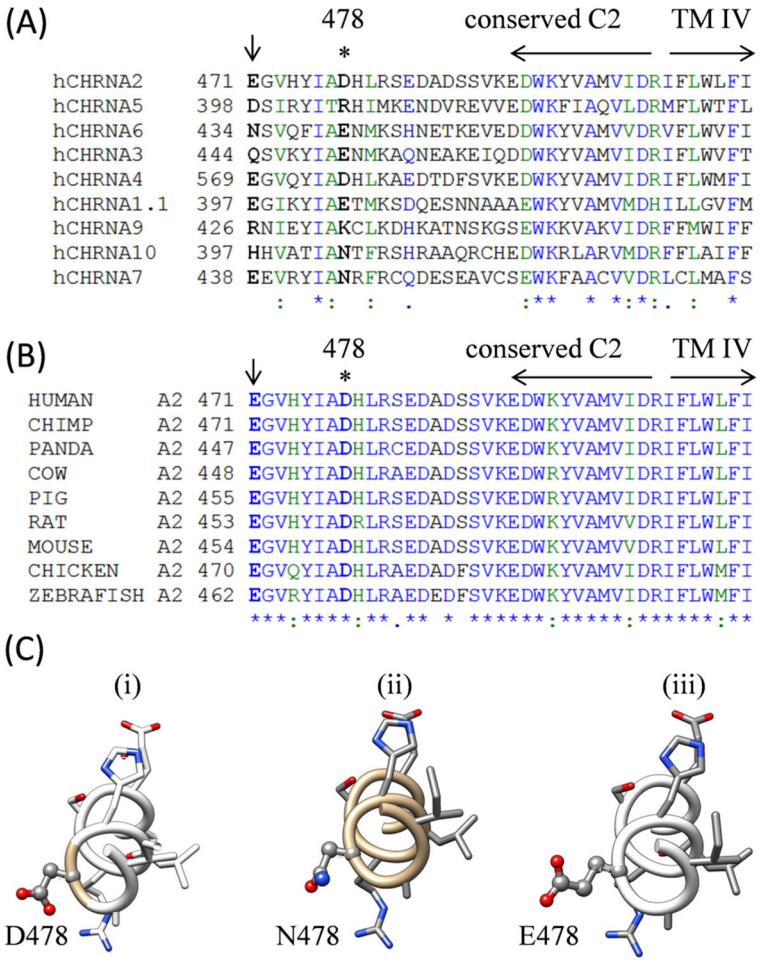

Figure 1. Bioinformatics analyses of the rare variations D478N and D478E that occur in human nAChR α2 subunit

(**A**) Protein sequences of human nAChR α subunits (α1-α7, α9-α10) are aligned using ClustalW. However, only in a portion of the alignment is shown here for the sake of clarity. (**B**) nAChR α2 subunits from several species: NM_000742.3 (Human), NM_001033935.1 (Chimpanzee), XM_002914435.1 (Panda), NM_001192710.1 (Cow), XM_003132824.1 (Pig), NM_133420.1 (Rat), NM_144803.2 (Mouse), NM_204815.1 (Chicken) and NM_001040327.1 (Zebrafish) are also aligned using ClustalW after translating their mRNA or genomic sequences to protein sequences. For both (**A**) and (**B**) numbering begins at translation start methionine of nAChR polypeptides and is shown in the region of interest. The location of the 478th residue (*; aspartate: D) of the human nAChR α2 subunit is shown relative to the 471st residue (↓) of the nAChR α2 subunit (which is equivalent to the 398th residue (↓) of the nAChR α5 subunit), subunit transmembrane domain IV and conserved region (MA/HA stretch) of the large cytoplasmic loop (C2). The 478th residue (*; D) of human nAChR α2 subunit has two rare alleles: asparagine (N) and glutamate (E). Symbols below the sequences indicate fully (*), strongly (:) and weakly (.) conserved residues. (**C**) Substitution of N (ii) or E (iii) for D (i) at the 478th residue of nAChR α2 subunit is shown graphically. Alpha helical structures are rendered using molecular graphics program (UCSF

Chimera) upon deriving a 3-D homology model of nAChR α2 subunit from the 3D-JIGSAW protein comparative modeling server.

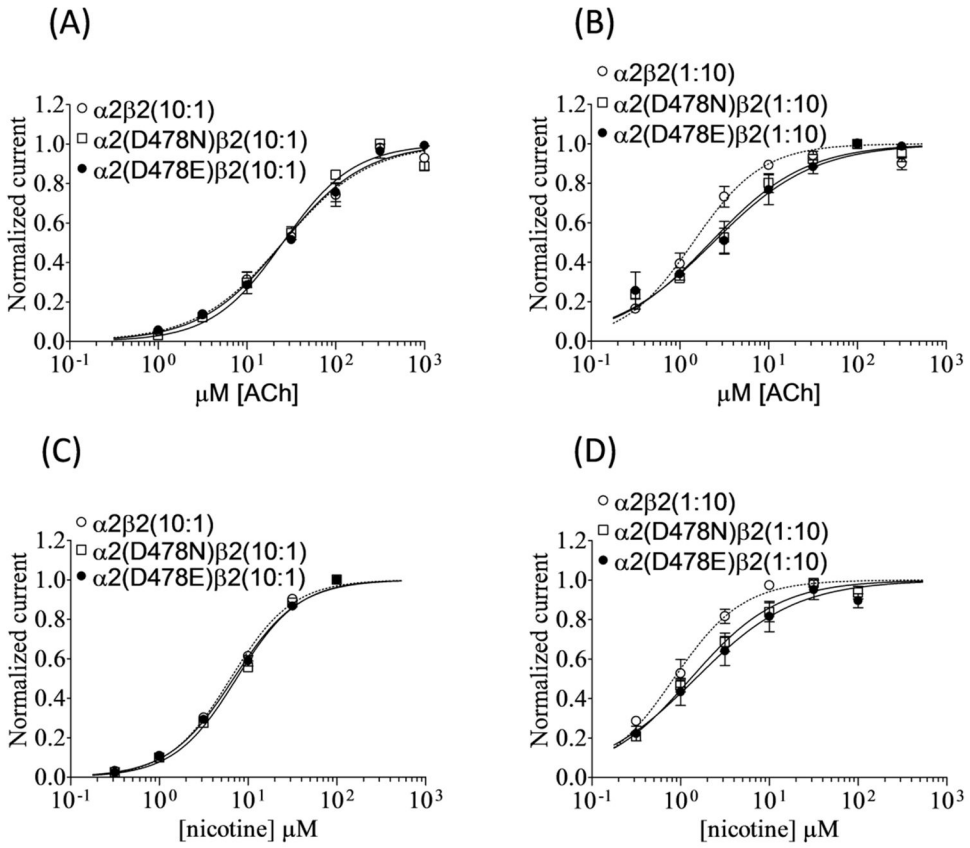

Figure 2. Effect of nAChR hα2 subunit rare variations, D478N and D478E, on the concentration-response (CR) relationship of WT α2β2-nAChRs expressed as LS (10:1) or HS (1:10) isoforms

Results averaged across experiments were used to produce concentration-response curves (ordinate – mean normalized current ± SE; abscissa – ligand concentration in log μM) for α2β2- (○), α2(D478N)β2- (□) or α2(D478E)β2- (●) nAChRs expressed in oocytes injected with 10:1 [**(A)** and **(C)**] or 1:10 [(**B**) and (**D**)] ratio of subunit (α:β) cRNAs for their responses to ACh [(**A**) and (**B**)] or nicotine [(**C**) and (**D**)] as indicated. CR curves indicate no change in agonist potency of LS or HS isoforms of α2β2-nAChRs as a result of either variation in nAChR hα2 subunit. Parameters for agonist action are summarized in Table 1.

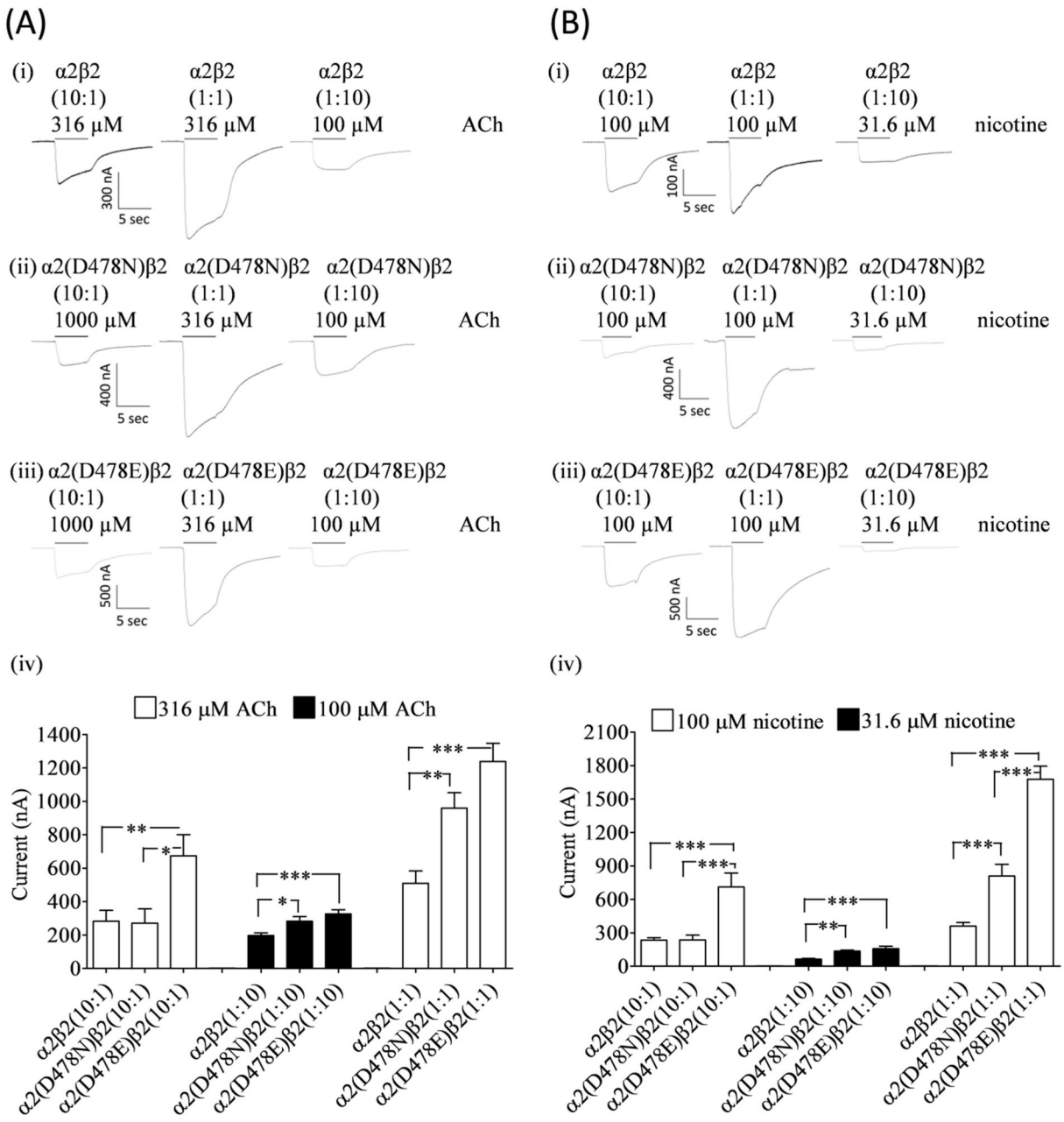

Figure 3. Comparison of peak current responses (I_{max}) of similar isoforms of α2β2-, α2(D478N)β2- and α2(D478E)β2-nAChRs

Representative traces for inward currents obtained in response to the application of most efficacious concentrations of ACh [(**A**) (i), (ii) and (iii)] or nicotine [(**B**) (i), (ii) and (iii)] (shown with the duration of agonist exposure as black bars above the traces) are shown for oocytes expressing indicated WT and variant α2β2-nAChRs and voltage clamped at −70 mV. Calibration bars are for 300, 400 or 500 nA (A), or 100, 400 or 500 nA (B) currents (vertical) and 5 sec (horizontal). Mean (± SE) I_{max} responses (n=6 to 23; Table 1) to indicated concentration of ACh [(**A**) (iv)] or nicotine [(**B**) (iv)] are compared between

similar isoforms of α2β2-, α2(D478N)β2- and α2(D478E)β2- nAChRs using one-way ANOVA with Tukey's post hoc comparison (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$).

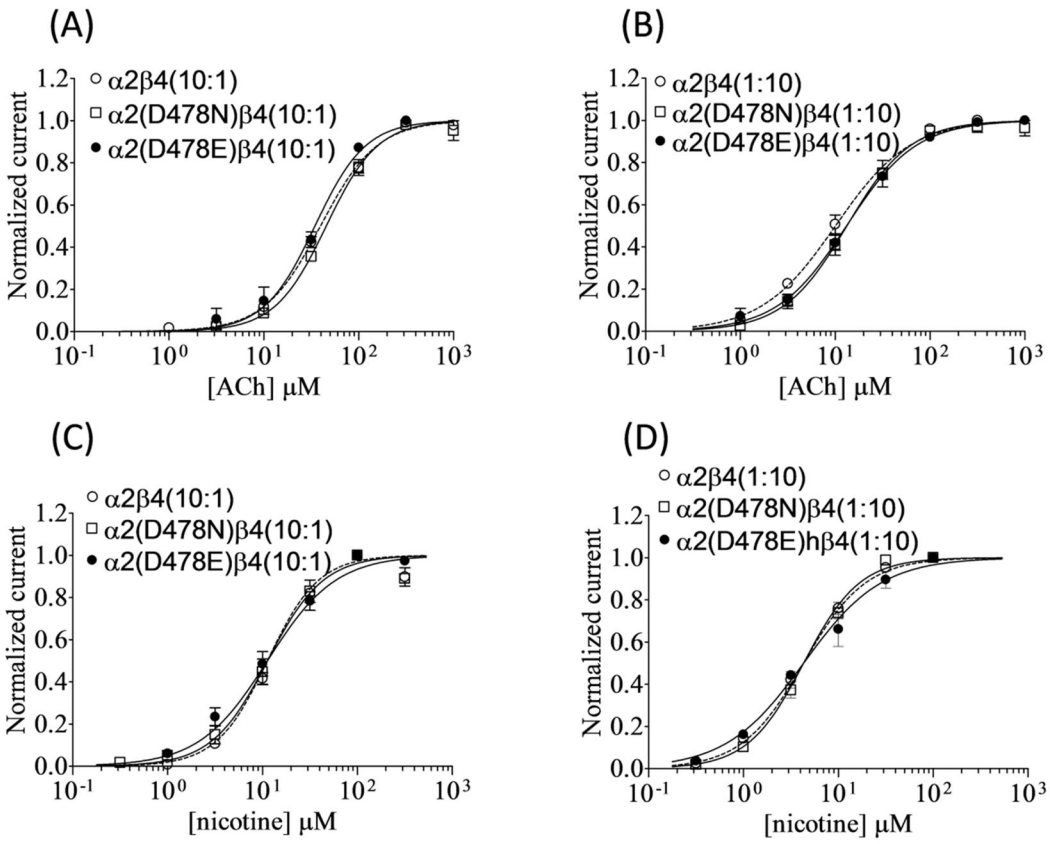

Figure 4. Effect of nAChR hα2 subunit rare variations, D478N and D478E, on the concentration-response (CR) relationship of WT α2β4-nAChRs expressed as LS (10:1) or HS (1:10) isoforms

Results averaged across experiments were used to produce concentration-response curves (ordinate – mean normalized current ± SE; abscissa-ligand concentration in log μM) for α2β4- (○), α2(D478N)β4- (□) or α2(D478E)β4- (●) nAChRs expressed in oocytes injected with 10:1 [(**A**) and (**C**)] or 1:10 [(**B**) and (**D**)] ratio of subunit (α:β) cRNAs for their responses to ACh [(**A**) and (**B**)] or nicotine [(**C**) and (**D**)] as indicated. CR curves indicate no change in agonist potency of LS or HS isoforms of α2β4-nAChRs as a result of either variation in nAChR hα2 subunit. Parameters for agonist action are summarized in Table 1.

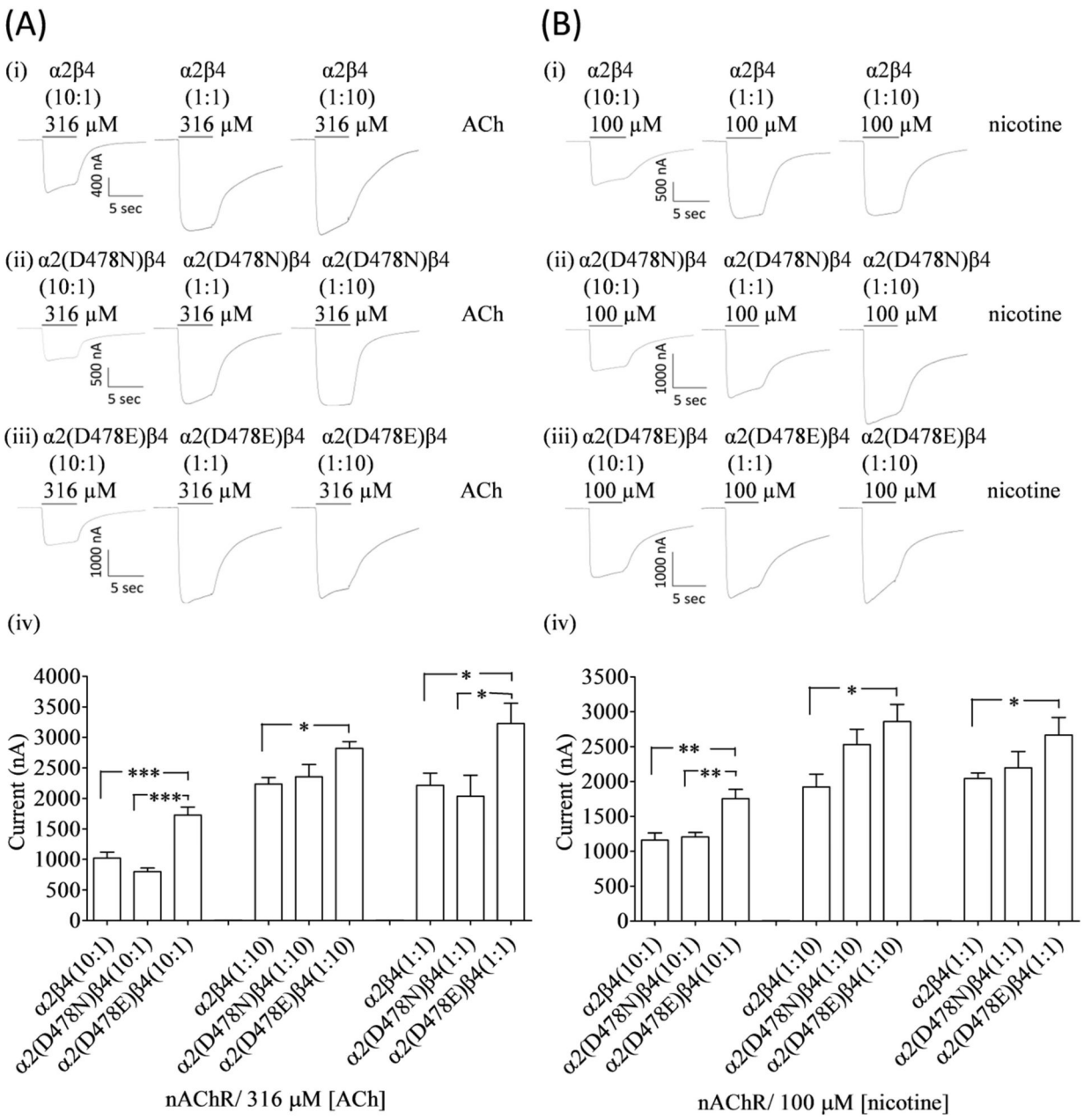

Figure 5. Comparison of peak current responses (I_{max}) between similar isoforms of α2β4-, α2(D478N)β4- or α2(D478E)β4-nAChRs

Representative traces for inward currents obtained in response to the application of most efficacious concentrations of ACh [(**A**) (i), (ii) and (iii)] or nicotine [(**B**) (i), (ii) and (iii)] (shown with the duration of agonist exposure as black bars above the traces) are shown for oocytes expressing indicated WT and variant α2β4-nAChRs and voltage clamped at −70 mV. Calibration bars are for 400, 500 or 1000 nA (**A**), or 500 or 1000 nA (**B**) currents (vertical) and 5 sec (horizontal). Mean (± SE) I_{max} responses (n=9 to 19; Table 1) to indicated concentration of ACh [(**A**) (iv)] or nicotine [(**B**) (iv)] are compared between

similar isoforms of α2β4-, α2(D478N)β4- or α2(D478E)β4- nAChRs using one-way ANOVA with Tukey's post hoc comparison (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$).

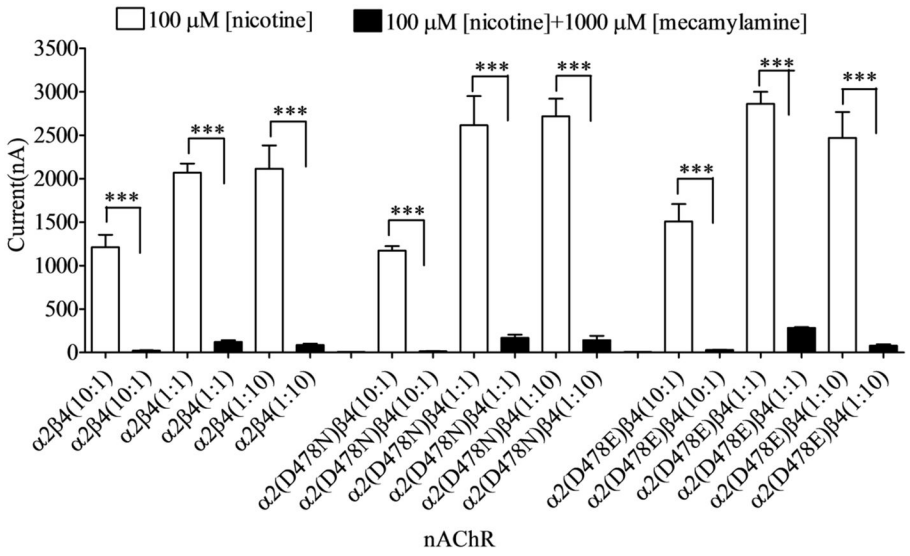

Figure 6. Mecamylamine inhibits nicotine induced peak current responses of LS (10:1), IS (1:1) and HS (1:10) isoforms of WT or variant α2β4-nAChRs

Nicotine (100 μM) induced peak current responses of α2β4, α2(D478N)β4- and α2(D478E):β4-nAChRs expressed in oocytes injected with 10:1 (n=11, 4 and 5 respectively), 1:1 (n=11, 5 and 3 respectively) or 1:10 (n=10, 4 and 5 respectively) ratio of subunit (α:β) cRNAs was significantly ($p < 0.001$) inhibited by 1000 μM mecamylamine. 'n' indicates numbers of individual oocytes tested. Data are presented as mean ± SE. Comparisons between control and mecamylamine treated isoforms are analyzed by Student's t test (two-tailed; ***, $p < 0.001$).

Table 1

Parameters for agonist action at human WT or variant α2*-nAChRs

Potencies (micromolar EC_{50} values and 95% confidence intervals), Hill coefficients (n_H ± SE), average (± SE) peak response (nA), and the concentration (μM) where I_{max} is achieved 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 (n) based on studies as shown in Figs. 2, 3, 4, 5 and S1. ↓ or ↑ indicates a significant ($p < 0.05$) decrease or increase, respectively, in the relevant parameter for nAChR containing α2(D478N) instead of WT α2 subunits. ↓ or ↑ indicates a significant ($p < 0.05$) decrease or increase, respectively, in the relevant parameter for nAChR containing α2(D478E) instead of WT α2 subunits. ▼ or ▲ indicates a significant ($p < 0.05$) decrease or increase, respectively, in the relevant parameter for nAChR containing α2(D478E) instead of α2(D478N) subunits. Numeric 'x' in the simple bracket indicates fold-change.

		Potency			Peak response		
Agonist	**nAChR subunit combinations**	**n**	**(μM) EC_{50} (95 % CI)**	**n_H±SEn_H**	**n**	**(nA) I_{max} (mean ± SE)**	**conc. I_{max} (μM)**
ACh	$1_{\alpha2\beta2(10:1)}$	5	26 (21–32)	0.9±0.08	23	283±64	316
	$1_{\alpha2\beta2(1:10)}$	6	1.4 (1.2–1.7)	1.1±0.1	13	196±17	100
	α2(D478N)β2(10:1)	3	23 (20–28)	1.1±0.09	9	271±86	316
	α2(D478N)β2(1:10)	3	2.2 (1.7–2.9)	0.8±0.08	11	282±29 ↑(1.4x)	100
	α2(D478E)β2(10:1)	3	27 (23–31)	0.9±0.06	11	674±126 ↑(2.4x) ▲(2.5x)	316
	α2(D478E)β2(1:10)	4	2.4 (1.7–3.3)	0.7±0.09	14	326±25 ↑(1.7x)	100
Nicotine	$1_{\alpha2\beta2(10:1)}$	3	6.3 (5.8–6.9)	1.2±0.06	16	235±20	100
	$1_{\alpha2\beta2(1:10)}$	5	0.8 (0.7–1)	1.1±0.13	15	63±6	31.6
	α2(D478N)β2(10:1)	4	7.4 (6.8–8.1)	1.2±0.18	12	237±42	100
	α2(D478N)β2(1:10)	3	1.3 (1–1.6)	0.9±0.08	12	135±9 ↑(2.1x)	31.6
	α2(D478E)β2(10:1)	3	6.8 (6.2–7.5)	1.2±0.05	9	711±125 ↑(3x) ▲(3x)	100
	α2(D478E)β2(1:10)	5	1.5 (1–2.2)	0.8±0.1	14	157±22 ↑(2.5x)	31.6
ACh	$1_{\alpha2\beta4(10:1)}$	7	40 (37–43)	1.5±0.07	9	1022±98	316
	$1_{\alpha2\beta4(1:10)}$	5	10 (8.6–12)	1.1±0.09	9	2235±108	316
	α2(D478N)β4(10:1)	3	45 (41–51)	1.6±0.1	10	800±60	316
	α2(D478N)β4(1:10)	4	13 (12–15)	1.3±0.09	9	2355±202	316
	α2(D478E)β4(10:1)	3	35 (29–41)	1.6±0.2	10	1727±132 ↑(1.7x) ▲(2.2x)	316
	α2(D478E) β4(1:10)	4	13 (12–15)	1.2±0.06	10	2821±110 ↑(1.3x)	316

		Potency			Peak response		
Agonist	**nAChR subunit combinations**	**n**	**(μM) EC_{50} (95 % CI)**	**n_H±SEn_H**	**n**	**(nA) I_{max} (mean ± SE)**	**conc. I_{max} (μM)**
Nicotine	[1]α2β4(10:1)	5	12 (11–14)	1.6±0.1	16	1159±104	100
	[1]α2β4(1:10)	5	4 (3.6–4.5)	1.3±0.08	19	1920±183	100
	α2(D478N)β4(10:1)	4	11 (9.3–13)	1.5±0.2	10	1205±63	100
	α2(D478N)β4(1:10)	3	4.6 (4.1–5)	1.5±0.09	10	2528±221	100
	α2(D478E)β4(10:1)	4	10 (8.6–12)	1.1±0.1	9	1753±134 ↑(1.5x) ▲(1.5x)	100
	α2(D478E)β4(1:10)	4	4.5 (3.7–5.5)	1.1±0.1	11	2858±246 ↑(1.5x)	100

[1]Part of control results are reported previously in *Neuropharmacology, 79:715–25.*