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A signal peptide missense mutation associated with nicotine dependence alters α2*-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) α2 subunit produces a threonine-to-isoleucine substitution (T22I) often associated with nicotine dependence (ND). We assessed effects on function of α2*-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 α:β subunit cRNAs injected) α2β2- or α2β4-nAChR and increased for putative high sensitivity (HS; 1:10 α:β subunit ratio) α2β2- (5.7–15-fold) or α2β4- (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 α2(T22I)β2-nAChR or for either α2*-nAChR subtype formed in the presence of equal amounts of subunit cRNA, slightly decreased for LS α2(T22I)β4-nAChR, but increased 1.4–2.4-fold for HS α2(T22I)β4-nAChR relative to receptors containing wild-type α2 subunits. These effects suggest that the α2 subunit SP mutation generally favors formation of LS receptor isoforms. We hypothesize that lower sensitivity of human α2*-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.

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 α subunits (α1–α10) or non-α subunits (i.e., β1–β4, γ, δ, ε subunits) (Lukas et al., 1999). In humans, nAChR other than the muscle-type (embryonic α1β1γδ- or adult α1β1γε-) are composed of different permutations of nine α (α1–α10) subunits and three β (β2–β4) 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 $GABA_A$, glycine and 5-HT_3A receptors, which collectively constitute the cys-loop superfamily of receptors (Lester et al., 2004; Sine and Engel, 2006). nAChR α subunits, but not non-α 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. α7*-, α4*-, α6*-, and α3*-nAChR subtypes but not α2*-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). α2*-nAChR are not as well studied and understood, partly because of restricted expression of α2 nAChR subunits (Ishii et al., 2005; Son and Winzer-Serhan, 2006; Wada et al., 1989). nAChR α2 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 α2 subunits/α2*-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 α2 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 α2 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 → T) missense mutation (rs2472553) in the SP of the human nAChR α2 subunit gene that leads to substitution of an isoleucine (I) residue for a threonine (T) residue at amino acid position 22 (i.e., T22I) 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; A**C**C → A**T**C) 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) 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 α2 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 (T22I or A125T) are not known but we got interested in the nAChR hα2 subunit SP mutation (T22I) 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 hα2*-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 hα2*-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 α2*-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) α2*-nAChRs. This is possible as subunit ratios could be varied (limiting one or other subunit) to drive the expression of LS- and HS- α2*-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) α2 subunits differ pharmacologically and functionally using two-electrode voltage clamp (TEVC) recordings. Results indicate that the SP mutation T22I modulates the function of both α2β2- and α2β4-nAChR. This is significant, because findings indicate that the SP mutation decreases sensitivities to nicotine and acetylcholine, likely by affecting subunit ratios in α2*-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 α (α1–α10) subunits or nAChR α2 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 α2 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 hydropathy 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 α2 mRNA in miRBASE (www.mirbase.org), TargetScan (www.targetscan.org), and snoRNA-LBME-db (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 $CaCl_2$, 1 $MgCl_2$, 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 α2, β2 and β4 subunits were subcloned into the oocyte expression vector pGEMHE as earlier described (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) (Dash et al., 2011a; Dash and Lukas, 2012). There was not the necessity to codon optimize the nAChR hβ4 subunit as α2β4-nAChR relatively highly functional. The mutation in the nAChR hα2 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 α2 subunit residue 22 are 5′-gtggctccttctgaAcccagcaggtggag-3′ (Forward, capitalization indicates the nucleotide changed from the wild-type) and 5′-ctccacctgctgggTtcagaaggagccac-3′ (Reverse). Identities of all wild-type (WT) or mutant subunits were confirmed by sequencing referenced to nucleotide/protein sequences available in GenBank.