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Rare Human Nicotinic Acetylcholine Receptor α4 Subunit (*CHRNA4*) Variants Affect Expression and Function of High-Affinity Nicotinic Acetylcholine Receptors[S]

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ABSTRACT

Nicotine, the primary psychoactive component in tobacco smoke, produces its behavioral effects through interactions with neuronal nicotinic acetylcholine receptors (nAChRs). $\alpha4\beta2$ nAChRs are the most abundant in mammalian brain, and converging evidence shows that this subtype mediates the rewarding and reinforcing effects of nicotine. A number of rare variants in the *CHRNA4* gene that encode the α4 nAChR subunit have been identified in human subjects and appear to be underrepresented in a cohort of smokers. We compared three of these variants (α4R336C, α4P451L, and α4R487Q) to the common variant to determine their effects on $\alpha4\beta2$ nAChR pharmacology. We examined [^{3}H]epibatidine binding, interacting proteins, and phosphorylation of the α4 nAChR subunit with liquid chromatography and tandem mass spectrometry (LC-MS/MS) in HEK 293 cells and voltage-clamp electrophysiology in *Xenopus laevis* oocytes. We observed significant effects of the α4 variants on nAChR expression, subcellular distribution, and sensitivity to nicotine-induced receptor upregulation. Proteomic analysis of immunopurified $\alpha4\beta2$ nAChRs incorporating the rare variants identified considerable differences in the intracellular interactomes due to these single amino acid substitutions. Electrophysiological characterization in *X. laevis* oocytes revealed alterations in the functional parameters of activation by nAChR agonists conferred by these α4 rare variants, as well as shifts in receptor function after incubation with nicotine. Taken together, these experiments suggest that genetic variation at *CHRNA4* alters the assembly and expression of human $\alpha4\beta2$ nAChRs, resulting in receptors that are more sensitive to nicotine exposure than those assembled with the common α4 variant. The changes in nAChR pharmacology could contribute to differences in responses to smoked nicotine in individuals harboring these rare variants.

Introduction

Nicotine, the principle psychoactive component of tobacco, exerts its effects through interactions with neuronal nicotinic acetylcholine receptors (nAChRs). The principal class of nAChRs that binds nicotine with high affinity in the mammalian central nervous system (CNS) is the $\alpha4\beta2$*-nAChR family (Picciotto et al., 1995; Millar and Gotti, 2009) (* designates potential additional subunits) (Lukas et al., 1999). Experiments with transgenic mice lacking β2* nAChRs (Picciotto et al., 1995, 1998), cell type-selective knockout of the α4 nAChR subunit (McGranahan et al., 2011), and knock-in mice with hypersensitive α4 nAChR subunits (Tapper et al., 2004, 2007) demonstrated that activation of $\alpha4\beta2$* nAChRs was necessary and sufficient for many behavioral effects associated with nicotine addiction. This has led recent genetic association studies to focus on nAChR subunit genes as targets of interest. A meta-analysis of nicotine dependence linkage studies supported significant genomewide linkage with a related trait at a chromosomal region overlying the *CHRNA4* locus (Han et al., 2010),

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ABBREVIATIONS: ACh, acetylcholine; ALS, amyotrophic lateral sclerosis; CNS, central nervous system; FT, flow through; HS, high sensitivity; LC-MS/MS, liquid chromatography and tandem mass spectrometry; LS, lower sensitivity; nAChR, nicotinic acetylcholine receptor; Phospho, phospho enriched; VAPB, vesicle-associated-protein-B.

and association studies found significant effects of several *CHRNA4* variants on nicotine dependence (Han et al., 2011; Kamens et al., 2013). A polymorphism in the α5 nAChR subunit gene (*CHRNA5*) (Bierut et al., 2008) has received particular attention, because it is consistently associated with nicotine dependence and produces an amino acid substitution (D397N) that results in altered function of $\alpha4\beta2\alpha5$ nAChRs (Bierut et al., 2008; Kuryatov et al., 2011).

Rare variants in *CHRNA4* also appear to be underrepresented among smokers (Xie et al., 2011). A particular rare variant (α4P451L) has also been associated with development of amyotrophic lateral sclerosis (ALS) (Sabatelli et al., 2009, 2012), suggesting that it may be of particular functional interest. In the current study, we characterize a subset of rare variants in the *CHRNA4* gene that alter amino acid sequence of the subunit. We chose polymorphisms based on a bioinformatic screen of predicted shifts in short linear interaction motifs relative to the wild-type subunit. We examined the effects of α4R336C, α4P451L, and α4R487Q on nAChR expression and protein/protein interaction in HEK 293 cells as well as receptor activation in *X. laevis* oocytes.

Materials and Methods

cDNA and Chemicals. Mutations corresponding to the identified rare variants were introduced into a human α4 cDNA (hα4) construct in a psp64-polyA vector (Promega, Madison, WI) using GeneArt Site-Directed Mutagenesis (Invitrogen, Carlsbad, CA). Except where noted, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). [^{3}H]Epibatidine (55.8 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Natick, MA)

Cell Culture and Transfection. HEK293 cells (ATCC 1573; American Type Culture Collection, Manassas, VA) were used for studies of receptor expression and identification of interacting proteins after transient transfection. Cells were allowed to reach ~90% confluence before transfection (typically ~96 hours) and harvested 24 hours after transfection. Details of cell culture conditions and transient transfection protocol are provided in the Supplemental Methods.

Quantitation of $\alpha4\beta2$ nAChR Expression with [^{3}H]Epibatidine Binding. Measurement of [^{3}H]epibatidine binding to cell membranes and solubilized receptors was performed as detailed in Supplemental Methods. Except where indicated, specific counts per minute were converted to femtomoles and normalized further to the total protein content present in each sample to provide units of femtomoles per milligramsprotein. Quantitation of plasma membrane $\alpha4\beta2$ nAChRs by cell surface biotinylation was performed as described previously (Kuryatov et al., 2005) with minor modifications. A detailed description is provided in the Supplemental Materials. Measurement of [^{3}H]epibatidine binding in subcellular compartments was achieved by fractionating transfected HEK293 cells with a subcellular fractionation kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Immunocapture of nAChRs and Identification of Receptor-Associated Proteins by Liquid Chromatography and Tandem Mass Spectrometry. Solubilized nAChRs were captured using M270 epoxy-coated Dynabeads (Invitrogen) coupled to 0.5 μg of mAb295/mg beads, which specifically recognizes β2-nAChRs (Whiting and Lindstrom, 1988), and the h$\alpha4\beta2$ nAChR-associated proteome was identified as described in the Supplemental Methods.

Expression and Recording of $\alpha4\beta2$ nAChRs in *X. laevis* Oocytes. Expression and electrophysiological recording of hα4(RV)β2 nAChRs in *X. laevis* oocytes was conducted essentially as reported previously (Williams et al., 2011). Agonist application was set at 8 seconds during recording, with 241-second washes in between stimulations. Responses to agonist are reported as percent of maximum response ± S.E.M. to limit interexperimental variation or differences in receptor expression because of effects of the hα4 polymorphisms related to nAChR assembly.

Data Analysis. For [^{3}H]epibatidine binding, kinetic parameters were determined by fitting the measured binding to a single-site hyperbolic saturation curve of the form $y = a[x]/b + [x]$, with measured binding (y) k_d (a) and B_{max} (b) at [ligand] x. Concentration-response effects on nicotine-induced nAChR upregulation were calculated with the equation $y = y0 + a[x]/b + [x]$, where binding (y) equals initial binding at [nicotine]0 ($y0$), nicotine concentration (x), maximal upregulation (b), and EC_{50} (a).

For electrophysiological studies, normalized response values were used to generate agonist concentration-response curves. The resulting curves were fit to two- and four-parameter regular hyperbolic equations followed by F-tests to determine the statistical preference for one or two distinct activation components, respectively. Curve fit equations were: $y = ax/b + x$, where (y) is recorded activity with maximal activation (a) and agonist EC_{50} (b) for a single component (2-parameter), and $y = (ax/b + x) + (cx/d + x)$, where ($y$) is recorded activity with high-sensitivity maximal activation (a), EC_{50} (b), and lower sensitivity (c) with EC_{50} (d) (4-parameter).

SPSS 21.0 and SigmaPlot 12.0 (2012 and 2011, respectively) were used for statistical analysis and data organization. Statistical significance was determined with one- or two-way analysis of variance or Student's t test, depending on the experimental design, with a confidence interval set at 95%.

Results

Effect of hα4 Variants on $\alpha4\beta2$ nAChR Expression. To identify any differences in efficiency of receptor assembly due to polymorphisms in the human α4 subunit (hα4, gene symbol *CHRNA4*), HEK293 cells were transfected with 1 μg of hβ2 cDNA per 250,000 cells and 0.01, 0.1, or 1 μg of hα4 cDNA, and binding of 2 nM [^{3}H]epibatidine was measured. [^{3}H]Epibatidine binding was dependent on the amount of hα4 cDNA added (Table 1). Transient transfection with large excesses of hα4 nAChR cDNA with a fixed concentration of hβ2 cDNA results in a sharp inverted-U relationship with respect to binding sites produced. To avoid this, we confined our analysis to the ratios of $\alpha4{:}\beta2$ cDNA that produced a proportional increase in binding sites per microgram α4 cDNA. The estimated 1/2 maximal cDNA concentration for expression did not vary across the α4 variants examined, but there was a significant effect of α4 variant on estimated maximal expression, with the α4P451L variant showing a 38% decrease in binding relative to control (Supplemental Fig. S1). All subsequent experiments with transient transfection of hα4 and hβ2 in HEK293 cells, including those for receptor upregulation, used a 1:1 ratio of $\alpha4{:}\beta2$ subunit cDNA.

HEK293 cells were transfected with hα4 and hβ2 cDNA and binding was measured across a range of [^{3}H]epibatidine concentrations (Table 2). There was a significant effect of hα4

TABLE 1
Calculated parameters for α4 cDNA-dependent [^{3}H]epibatidine binding site production

hα4 Variant	Max Expression	1/2max [cDNA]
	fmol/mg	*μg/250 K cells*
Common	368.80 ± 11.20	0.21 ± 0.02
α4R336C	408.20 ± 30.20	0.30 ± 0.07
α4P451L	229.90 ± 26.50*	0.37 ± 0.13
α4R487Q	534.70 ± 22.50*	0.41 ± 0.05

*$P < 0.05$ compared to the common variant.

variant on B_{max}. Dunnett's post hoc analysis revealed that there is an approximate twofold decrease in the estimated B_{max} of the α4P451L variant for [^{3}H]epibatidine binding relative to control but no difference in the estimated affinity of any of the hα4 variants for [^{3}H]epibatidine (Supplemental Fig. S2; statistics for all [^{3}H]epibatidine binding experiments are listed in Supplemental Section 1).

Effects of hα4 Variants on $\alpha4\beta2$-nAChR Upregulation and Plasma Membrane Levels in Response to Nicotine Exposure. $\alpha4\beta2$ nAChRs are upregulated by long-term exposure to nicotine (reviewed by Lester et al., 2009). To test for sensitivity and extent of nicotine-induced receptor upregulation, nicotine (0, 3, 10, 30, 100, 1000, and 3000 nM) was added to the wells with hα4 and hβ2 cDNAs, and the cells were incubated overnight. After 24-hour nicotine exposure, all hα4 variants showed concentration-dependent and saturable upregulation of [^{3}H]epibatidine binding sites (Fig. 1A; Table 3). There was no significant difference in either maximal upregulation by nicotine or EC_{50}. Converting the data from femtomoles per milligram to percentage of control (no nicotine) to produce a measure of fold-increase reveals significant differences in the ability of nicotine to upregulate $\alpha4\beta2$ nAChRs depending on the hα4 variant (Fig. 1B). Dunnett's post hoc analysis shows that nicotine upregulates [^{3}H]epibatidine binding in α4P451Lβ2-transfected HEK293 cells to a greater extent than nAChRs, incorporating the other α4 variants examined with no significant effect on EC_{50} (Table 4).

To determine the distribution of [^{3}H]epibatidine binding sites in cells, surface receptors were biotinylated prior to cell lysis, and binding to biotinylated receptors was quantified. Measuring [^{3}H]epibatidine binding prior to solubilization in phosphate-buffered saline supplemented with 2% Triton X-100 quantitates all available binding sites (including immature and intracellular pools of receptors), whereas avidin capture of biotinylated receptors from detergent extracts enables detection of plasma membrane nAChRs. Under control conditions, there are fewer total α4P451Lβ2 nAChRs compared with the other variants (Fig. 2A), but similar levels of plasma membrane binding sites (Fig. 2C). The deficient expression of [^{3}H]epibatidine binding in cells transfected with α4P451L does not reflect a deficit in cell surface α4P451Lβ2 nAChRs. After treatment with 1 μM nicotine for 24 hours, significant upregulation of [^{3}H]epibatidine binding sites was observed in both total and surface receptor pools of all α4 variants (Fig. 2C; Table 5). As observed with surface expression at baseline, the extent of upregulation measured as fold change relative to control was much greater in nAChRs containing the hα4-P451L variant (Fig. 2, B and D, statistical test results are presented in Supplemental Section 2).

TABLE 2
Calculated parameters for saturation of [^{3}H]epibatidine binding

hα4 Variant	B_{max}	K_d
	fmol/mg	*pM*
Common	560.0 ± 17.0	26.8 ± 7.0
α4R336C	595.0 ± 57.0	31.0 ± 8.0
α4P451L	291.2 ± 10.9*	16.9 ± 5.2
α4R487Q	675.0 ± 13.0	24.1 ± 1.4

*$P < 0.05$ compared to the common variant.

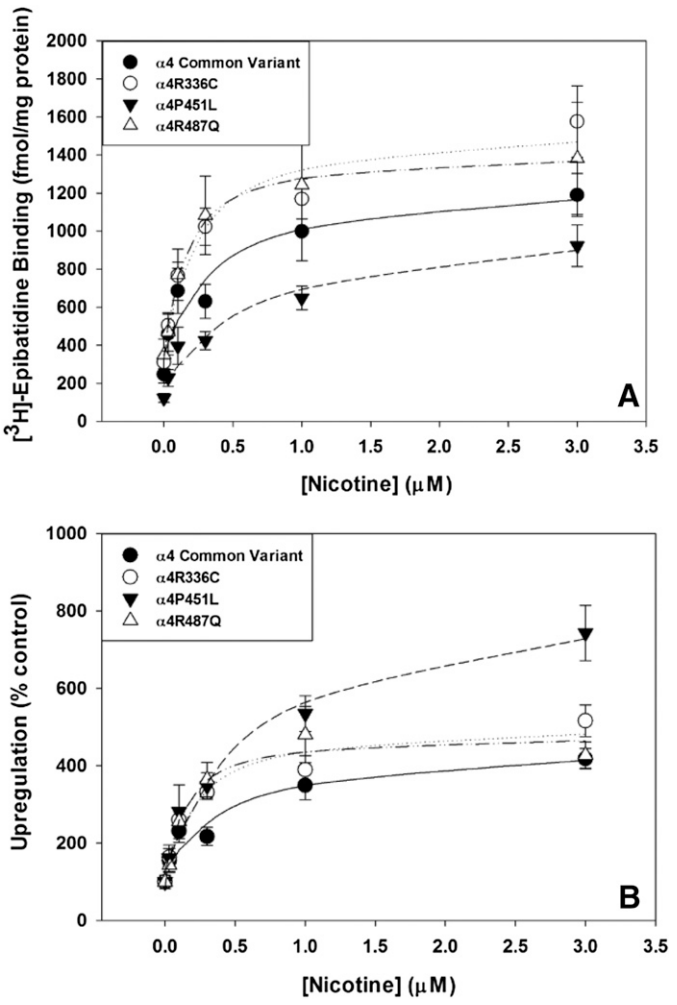

Fig. 1. Effects of nicotine on levels of nAChRs incorporating α4 variants. (A) Nicotine upregulates expression of [^{3}H]epibatidine binding sites for the α4 common variant (●), α4R336C (○), α4P451L (▼) and α4R487Q (△) with equivalent potency and maximal expression. (B) Nicotine-induced upregulation of [^{3}H]epibatidine binding sites as a percent of control reveals that the level of upregulation compared with baseline is greater for α4P451L (▼) and results in greater maximal upregulation compared with the common variant (●), α4R336C (C) and α4R487Q (△).

Effects of hα4 Variants on Associated Proteins and Phosphorylation Patterns Identified by Tandem Mass Spectrometry. Eluted $\alpha4\beta2$ nAChR complexes were enriched for phosphorylated peptides prior to LC-MS/MS analysis to split each group into phospho-enriched (Phospho) and flow-through (FT) sets, and both sets were analyzed to identify phosphorylated residues of peptides in the MS/MS spectra. We hypothesize that because protein kinases and phosphatases target specific residues in the M3-M4 loop (Pollock et al., 2009), the rare variants examined in this study might affect the accessibility of these sites. From the initial lists of identified proteins for each hα4 variant, we used a protein-exclusion process to refine the apparent interactomes. Obvious contaminant proteins (keratins, dermicidins, hornerins) were excluded. Because the nAChRs were isolated from solubilized protein extracts prepared from whole cells, some nuclear and mitochondrial proteins were also identified that would normally not be expected to interact with $\alpha4\beta2$ nAChRs, and these proteins were also excluded from further consideration.

TABLE 3
Calculated parameters for nicotine-induced upregulation of [^{3}H] epibatidine binding sites

hα4 Variant	Max Upregulation	Nicotine EC_{50}
	fmol/mg	*μM*
Common	1262.34 ± 122.83	1.55 ± 0.66
α4R336C	1154.67 ± 87.67	0.33 ± 0.15
α4P451L	986.74 ± 218.29	0.77 ± 0.34
α4R487Q	1197.21 ± 233.23	0.20 ± 0.06

These "culled" protein lists were compared across hα4 variants to generate sets of proteins common to all α4 variants, unique to each hα4 variant, and sets where proteins were common to three of four variants and conspicuously absent from the fourth. These lists were compiled for each fraction (Phospho or FT). The complete sets of interacting proteins identified for each variant are available at http://yped.med.yale.edu/repository/. Protein discrepancies are evident for α4P451L in particular. A number of 14-3-3 chaperone protein isoforms are not identified by LC-MS/MS from immunopurified α4P451Lβ2 nAChRs that are repeatedly identified in purified α4β2 nAChRs containing the other hα4 variants. Several importin isoforms are present in α4P451Lβ2 complexes identified by LC-MS/MS that are absent in the profiled complexes of the other hα4 variants [discrepancies for each variant are listed in Supplemental Tables S1 (Phospho) and S2 (FT)]. These results provide evidence of aberrant protein/protein interactions attributed to a significant shift in the conformation of the M3-M4 loop in the α4P451L variant that disrupts its association with commonly observed nAChR chaperones and recruits importin isoforms normally associated with nuclear transport. Therefore, we examined the subcellular distribution of [^{3}H]epibatidine binding sites in HEK 293 cells transfected with hβ2 and either hα4 or hα4P451L. Transfected cells were processed into cell membrane (plasma membranes and endoplasmic reticulum), nuclear, and cytoskeletal fractions, and [^{3}H]epibatidine bindine was assessed in each sample. Nicotinic binding sites in the nuclear fractions of transiently transfected cells were not altered by inclusion of the α4P451L variant (Supplemental Fig. S3), indicating that the association of importin isoforms with α4P451L does not result in nuclear import of α4β2 nAChRs but instead reflects inappropriate protein/protein interactions that could be expected to influence receptor assembly and post-translational modification.

Analysis of the 270-amino acid sequence of the M3-M4 intracellular loop of the hα4 nAChR subunit with NetPhos 2.0 (www.cbs.dtu.dk) identifies 17 potential serine phosphorylation sites. None of the α4 variants examined in this study are predicted to affect any of these 17 sites; however, there was a significant discrepancy in the phosphorylation patterns of the identifiable serine residues across the four α4 variants. Consistent with previous studies (Pollock et al., 2007), we observed phosphorylation of serine residues only on the M3-M4 loop of the α4 subunit (Table 6). Proteolysis of the hα4 subunit by trypsin and LysC prior to LC-MS/MS restricts the absolute coverage of the receptor by producing peptide fragments too small to be unambiguously assigned, and so we anticipated missing 8 of 17 possible phosphorylation sites. There was no difference in the peptide sequence coverage of the M3-M4 loop for the common hα4 variant compared with the α4R336C and α4R487Q variants, so differences in phosphorylation state of identified serine residues are not likely because of differential sequence coverage. The identification of the phosphorylation state of serines in the M3-M4 loop of α4P451L was not possible, because its coverage was insufficient for effective analysis.

TABLE 4
Nicotine-induced upregulation expressed as fold change relative to control

hα4 Variant	Max Upregulation	Nicotine EC_{50}
	Fold change	*μM*
Common	4.5 ± 0.5	1.59 ± 0.70
α4R336C	4.1 ± 0.4	0.34 ± 0.20
α4P451L	8.5 ± 1.3*	1.24 ± 0.41
α4R487Q	4.0 ± 0.4	0.20 ± 0.06

*$P < 0.05$ compared to the common variant.

Electrophysiological Characterization of α4 Variants. We expressed hα4 variants α4R336C, α4P451L, and α4R487Q with human β2 in *X. laevis* oocytes and recorded currents elicited by application of nAChR agonists. With equal amounts of hα4 and hβ2 cRNA, activation of common variant α4β2 with ACh produced the predicted biphasic concentration-response curve (Table 7), indicating the presence of nAChRs with high sensitivity to ACh (HS) and a lower sensitivity component (LS), as has been described previously (Marks et al., 2010). The other hα4 variants (α4R336C, α4P451L, α4R487Q) all had ACh concentration-response curves that favored the activation of a single HS component. The calculated potency of ACh for HS α4β2 activation was similar across variants, indicating that these variants in the α4 subunit do not appreciably affect sensitivity to activation by ACh but limit the stoichiometry of assembled nAChRs to the HS form of $\alpha4_2\beta2_3$. Nicotine produces similar concentration-response relationships as ACh (Table 8), with the exception that the HS form of common variant α4β2 is more prominently activated, with no change in potency, when nicotine is used as the agonist. (Calculated activation parameters are found in Tables 7 and 8; Fig. 3.)

Because the subunit composition and stoichiometry of α4β2 nAChRs affects their functional profiles, we expressed the α4 variants as concatamers (α4-β2 dimers) to isolate the two defined stoichiometries and determine whether any effects of α4 variant were apparent when receptor assembly is experimentally constrained. Agonist-evoked responses from α4β2 nAChRs assembled with concatenated subunits (as described in Zhou et al., 2003) largely confirmed our hypothesis that hα4 rare variants will affect receptor assembly. In particular, α4P451L shows a reduced ability to assemble as an LS receptor [$\alpha4_3\beta2_2$] even when introduced into a concatamer, and α4R336C forces assembly of both HS and LS stoichiometries under every condition examined (full concentration-response curves and parameters of concatenated receptor experiments are found in Supplemental Figs. S4 and S5, and Table S3, respectively; a detailed description of receptor concatamer experiments is presented in Supplemental Materials).

Effects of 24-hour Nicotine Exposure on Activation Parameters of α4 Variants. Prolonged nicotine exposure promotes the production of HS nAChRs in heterologous systems expressing human α4β2 nAChRs (Kuryatov et al., 2005). Because the α4 variants appear to alter the

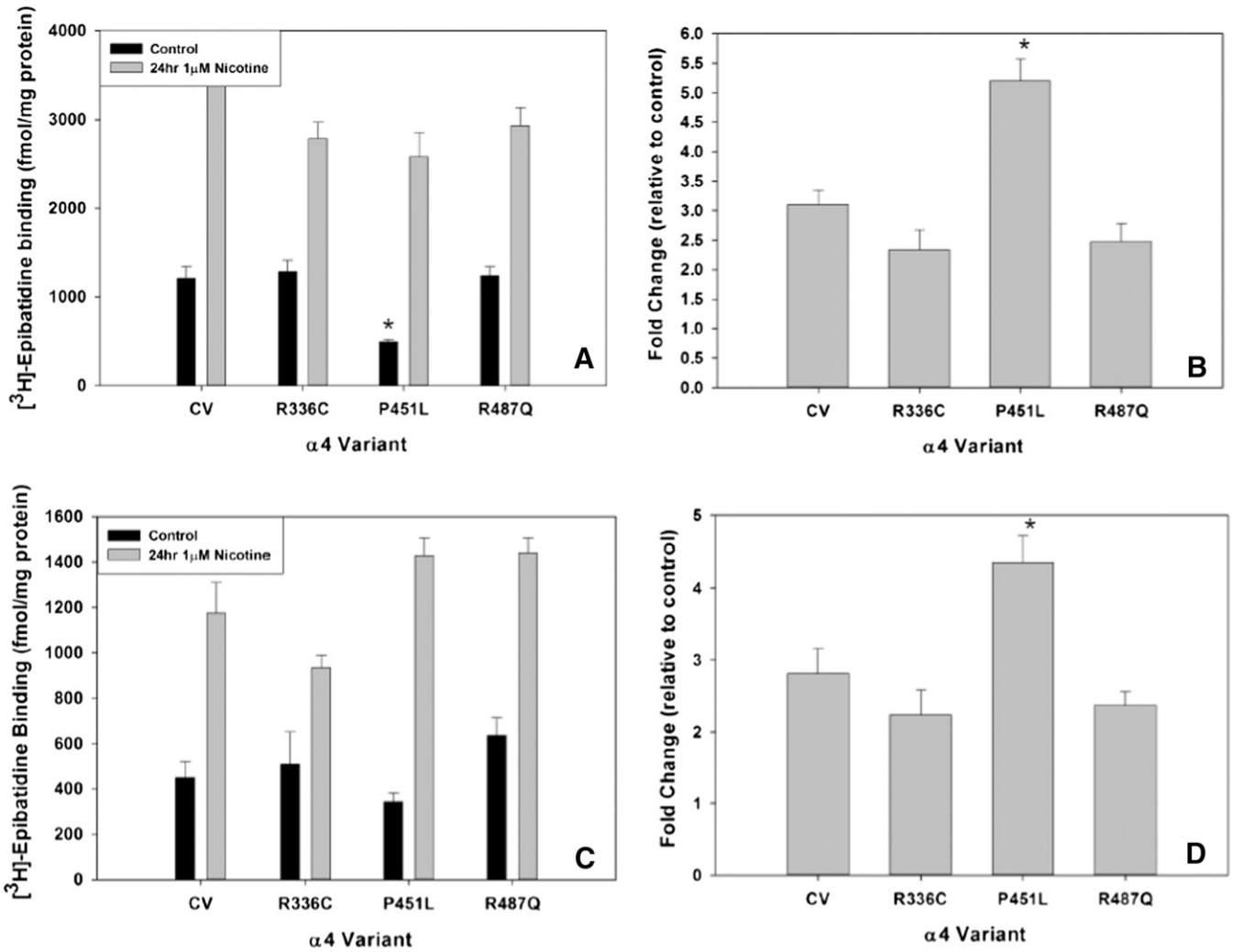

Fig. 2. Cell surface expression of nAChRs incorporating $\alpha 4$ variants. (A) Total cell membrane expression of [^{3}H]epibatidine binding sites ±24-hour exposure to 1 μM nicotine. Under control conditions (black bars) α4P451L has significantly lower total [^{3}H]epibatidine binding that was rescued as compared with the other variants after 24-hour incubation with 1 μM nicotine (gray bars). (B) Total cell membrane upregulation induced by 24-hour exposure to 1 μM nicotine expressed as fold change relative to control indicates that the α4P451L variant is upregulated to a greater extent than the other variants. (C) Cell surface levels of [^{3}H]epibatidine binding sites ± 24-hour nicotine exposure shows that despite the significant difference in total binding site expression, surface levels of α4P451L are normal under control conditions (black bars) and are strongly upregulated after nicotine treatment (gray bars). (D) Cell surface upregulation induced by 24-hour 1 μM nicotine expressed as fold change relative to control indicates that α4P451L enhances surface expression of nAChRs after nicotine treatment to a greater degree than the common variant, α4R336C, and α4R487Q. *$P < 0.05$ compared to the common variant.

stoichiometry of $\alpha 4\beta 2$ nAChRs, we examined $\alpha 4\beta 2$ nAChR activation by ACh and nicotine after oocytes were incubated with 1 μM nicotine for 24 hours. The effects of nicotine exposure were strikingly divergent according to hα4 variant (Fig. 4A; Tables 9 and 10). Consistent with previous reports, nicotine incubation shifted the concentration-response curve for ACh to a single HS component for common variant $\alpha 4\beta 2$ nAChRs, indicating that exposure to nicotine (1 μM) favors the production of $\alpha 4\beta 2$ nAChRs with the $\alpha 4_2\beta 2_3$ stoichiometry. In contrast, nicotine exposure shifted the potency of ACh to a significant degree in the α4R336C variants, resulting in the appearance of a substantial LS response profile. This

TABLE 5
Effects of 1.0 μM nicotine on plasma membrane and total binding sites

hα4 Variant	Control Total Binding	Control Surface Binding	Control	Nicotine Total Binding	Nicotine Surface Binding	Nicotine
	fmol/mg		*% surface binding*	*fmol/mg*		*% surface binding*
Common	1205 ± 138	450 ± 70	37.5 ± 3.7	3592 ± 197	1174 ± 136	32.4 ± 2.4
α4R336C	1281 ± 127	511 ± 142	43.0 ± 13.8	2784 ± 186*	936 ± 54	34.7 ± 3.9
α4P451L	491 ± 24*	342 ± 41	69.2 ± 6.3*	2581 ± 272*	1428 ± 77	57.6 ± 12.3
α4R487Q	1242 ± 97	636 ± 79	51.8 ± 5.4	2932 ± 204	1440 ± 66	51.2 ± 6.6

*$P < 0.05$ compared to the common variant.

TABLE 6
Phosphorylated residues identified on α4 nAChR subunits by MS/MS

Identified Residue	α4-Common Variant	α4 R336C	α4 P451L	α4 R487Q	Putative Kinases
Ser374	Yes	Yes	ND	No	BARK
Ser467	Yes	Yes	ND	Yes	CamKII, MAPKI, PKA
Ser472	No	No	ND	Yes	CDK5, GSK3
Ser473	Yes	No	ND	No	CKII
Ser488	Yes	Yes	ND	No	AKTK, MAPKI
Ser527	Yes	Yes	ND	No	CDK5, GSK3
Ser539	No	Yes	ND	Yes	GSK3, MAPKII
Ser541	Yes	Yes	ND	Yes	CKI, ERKI, ERKII, CDK5
Ser561	No	Yes	ND	Yes	CDK5, CKI, ERKI, ERKII, GPCRK, GSK3, MAPKII

ND, not determined.

effect appears to reflect the preferential assembly of LS α4R336Cβ2 nAChRs after prolonged nicotine exposure, an effect in striking contrast to what is routinely observed with the common variant of hα4. Nicotine exposure did not affect the ACh concentration-response profile for α4P451L, apart from a slight decrease in the overall magnitude of the response (likely due to nicotine-mediated desensitization). The α4R487Q variant retained both HS and LS components after 24-hour nicotine exposure, indicating a decrease in the preferential assembly of HS α4β2 nAChRs in the presence of nicotine. Both control and P451L α4 variants maintained a single HS concentration-response profile in response to acute nicotine challenge after nicotine exposure, whereas the R336C and R487Q α4 variants showed the same biphasic responses as observed before 24-hour nicotine exposure (Fig. 4B). Incubation of oocytes transfected with the α4R336C variant with nicotine for 24 hours had the same effect on nicotine-evoked currents as observed with ACh, revealing a shift in agonist potency that produced a large LS response. Representative traces for agonist-evoked inward currents through α4R336Cβ2 and common variant α4β2 nAChRs after 24-hour nicotine exposure are shown in Fig. 5. These results show that whether ACh (Fig. 5A) or nicotine (Fig. 5B) is used as the agonist, α4R336Cβ2 nAChRs are substantially less sensitive to agonist activation after 24-hour nicotine exposure than common variant α4β2 nAChRs.

Discussion

We examined the effects of rare *CHRNA4* variants on expression, protein/protein interactions, and activation parameters of α4β2 nAChRs. We observed significant effects of these rare α4 variants on nAChR binding site expression and upregulation by nicotine. Most importantly, the α4 variants also altered the nAChR interactome and the parameters of α4β2 nAChR activation by ACh and nicotine and resulted in significant shifts in concentration-response profiles both under control conditions and after a 24-hour exposure to nicotine, indicating that smoking could alter the properties of α4β2 nAChRs in humans carrying these rare genetic variants.

nAChR subunits all possess a large intracellular loop between the M3 and M4 transmembrane domains. The α4 nAChR subunit has the largest (270-amino acids long) M3-M4 loop, and this domain is pivotal in nAChR assembly and functional modulation (Harkness and Millar, 2002; Kuo et al., 2005; Kracun et al., 2008; Tsetlin et al., 2011). The loop is predicted to be largely unfolded, existing as a series of linear interaction motifs (Kukhtina et al., 2006). The α4 polymorphisms examined in this study (R336C, P451L, R487Q) all occur within the M3-M4 loop at amino acid residues that are 100% conserved across humans, mice, rats, and chickens, suggesting that these rare variants will affect one or more elements of α4β2 nAChR maturation, expression, and function.

Maximal nAChR production was significantly affected by α4 variant: the α4R487Q variant produced significantly more [^{3}H]epibatidine binding sites when cDNA concentration was maximal, and α4P451L produced significantly fewer nicotinic binding sites. The α4R336C polymorphism was equivalent to the common variant under these conditions. The estimates for α4 cDNA-dependent α4β2 nAChR production were confined to ratios of α4:β2 that yielded concentration-dependent increases in binding sites, as extreme ratios of α4:β2 can produce sharp declines in receptor assembly. Although this restricted curve does not show the absolute saturation of binding site production as a function of α4 cDNA concentration, it does provide accurate estimates of the differences in binding sites produced across the α4 variants examined. Eliminating ER retention motifs in the β2 nAChR enhances nAChR transport from the ER, and the effect of the α4R487Q variant is consistent with accelerated nAChR assembly because of enhanced ER transport (Srinivasan et al., 2011), considering the proximity of this variant to an established RxR retention motif in the hα4 subunit. The α4P451L variant introduces a peroxisome targeting motif (PTS) and eliminates a highly conserved (100% across humans, rats, mice, and chickens) diproline motif. The decrease in [^{3}H]epibatidine

TABLE 7
Calculated parameters for acetylcholine concentration-response profiles

hα4 Variant	HS EC_{50}	HS Max Response	LS EC_{50}	LS Max Response
	μM		μM	
Common	2.12 ± 0.34	0.89 ± 0.06	109.0 ± 41.0	0.47 ± 0.05
α4R336C	4.34 ± 1.06	1.14 ± 0.04	—	—
α4P451L	5.30 ± 1.20	1.08 ± 0.04	—	—
α4R487Q	11.2 ± 5.1	1.28 ± 0.10	—	—

TABLE 8
Calculated parameters for nicotine concentration-response profiles

hα4 Variant	HS EC_{50}	HS Max Response	LS EC_{50}	LS Max Response
	μM		μM	
Common	0.73 ± 0.24	0.37 ± 0.02	—	—
α4R336C	0.93 ± 0.29	0.29 ± 0.02	—	—
α4P451L	0.97 ± 0.32	0.26 ± 0.01	—	—
α4R487Q	2.88 ± 0.82	0.49 ± 0.03	—	—

binding sites observed with α4P451L is consistent with the possibility that introducing a PTS results in mistargeting of α4 subunits, limiting the total number of pentameric α4β2 nAChRs produced. Although other interpretations are possible, the current observations provide a context for future studies. The α4R336C variant lost a putative 14-3-3 binding motif, but had no effect on the concentration dependence for

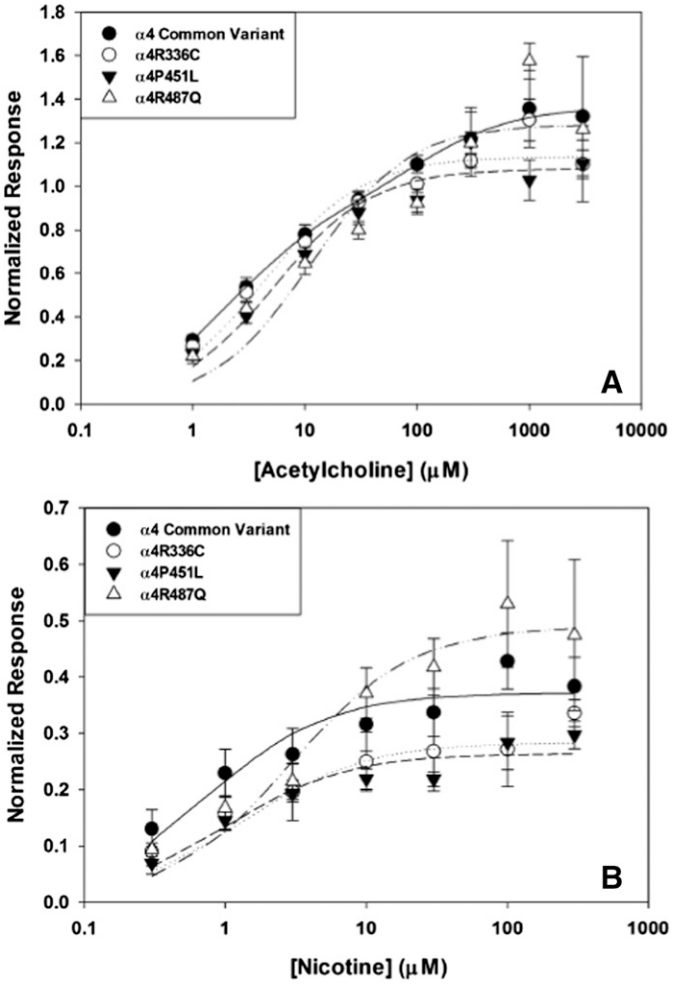

Fig. 3. Electrophysiological recordings of α4β2 nAChRs expressed in *X. laevis* ooctyes and activated by acetylcholine. (A) The common variant of α4 (●) demonstrates a biphasic concentration-response curve, indicative of high- and low-sensitivity activation components. α4R336C (○), α4P451L (▼), and α4R487Q (△) all show monophasic, high-sensitivity activation by acetylcholine. (B) Electrophysiological recordings of α4β2 nAChRs expressed in *X. laevis* oocytes activated by nicotine. The common variant of α4 (●) has a monophasic, high-sensitivity concentration-response relationship with nicotine. Similarly, α4R336C (○), α4P451L (▼), and α4R487Q (△) also show monophasic high-sensitivity activation by nicotine.

cDNA effects on nAChR production, which was somewhat unexpected, given the role of 14-3-3 binding in nAChR trafficking (Jeanclos et al., 2001). Interestingly, despite these differences in assembly across variants, the number of α4β2 nAChRs that were expressed on the cell surface was not affected by any of these α4 variants.

Upregulation of α4β2* nAChRs by long-term exposure to nicotine has been suggested to play a role in the development/maintenance of nicotine dependence and has been observed repeatedly both in vitro and in vivo in rodents and human smokers (Staley et al., 2006; Lester et al., 2009). Nicotine increased [^{3}H]epibatidine binding to nAChRs containing all α4 variants examined; however, although production of α4β2 nAChRs is deficient for α4P451L under control conditions, incubation with nicotine effectively "normalized" its ability to produce [^{3}H]epibatidine binding sites with identical parameters as the other α4 variants.

Upregulation of nAChRs has been attributed to the ability of nicotine to bind to and stabilize nascent nAChRs in the process of assembly and maturation in the ER and reduce nAChR degradation after insertion into the plasma membrane (Kuryatov et al., 2005; Srinivasan et al., 2011). This process of pharmacological chaperoning is thought to involve selective stabilization of fully folded nAChR intermediates, rather than to promote the folding of primarily linear peptides (Lester et al., 2009). The observation that the α4P451L variant produces substantially fewer nAChRs, but that pharmacological chaperoning with nicotine boosts α4P451L assembly with β2 to levels consistent with the α4 common variant is particularly interesting because the P451L substitution removes a highly conserved diproline residue and introduces a novel PTS motif that may mistarget the receptor to the peroxisome or other intracellular compartments. It is possible that a conformational change produced when nascent α4P451Lβ2 receptors bind nicotine obscures the recognition of the PTS motif and allows assembly to proceed normally, without mistargeting or preferentially degrading the α4P451Lβ2 receptor intermediates. An alternate possibility is that elimination of the diproline residue produces an α4 subunit with more conformational flexibility in the large M3-M4 loop than the common variant and may trigger ER-resident chaperoning in a manner that favors rapid degradation in the absence of a stabilized conformation (as would be the case with pharmacological chaperoning by nicotine). Pro-pro motifs tend to produce segments of polypeptides with a constrained conformation (Saha and Shamala, 2012), so eliminating a strong structural constituent motif would be expected to radically alter the position and accessibility of several linear interaction motifs both C- and N-terminal of the disrupted sequence.

The possibility that the α4P451L variant results in ER-dependent nAChR degradation is supported by studies that identified a significant association between several rare variants in the α4 nAChR subunit gene, in particular the α4P451L polymorphism, and ALS (Sabatelli et al., 2009, 2012). A study examining an ALS-risk variant of the β4 nAChR subunit (β4R348C) found decreased expression of nAChRs when this variant was coexpressed with hα4, an effect primarily due to decreased ER export of α4β4R348C nAChRs (Richards et al., 2011). In that study, submicromolar nicotine did not alter the amount of α4β4 plasma membrane expression, so the deficient nAChR production resulting from expression of the β4R348C

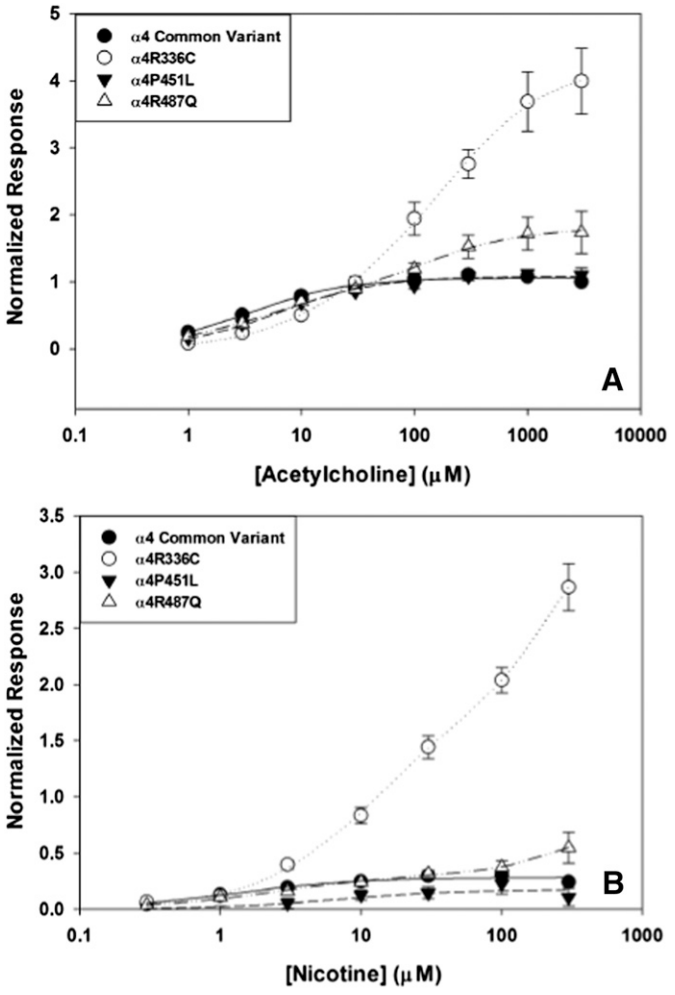

Fig. 4. Electrophysiological recordings of $\alpha4\beta2$ nAChRs expressed in *X. laevis* oocytes activated by acetylcholine following 24 hour exposure to 1 μM nicotine. (A) The concentration-response curve for the common $\alpha4$ variant (●) shifts from biphasic to monophasic, high-sensitivity activation by acetylcholine. The concentration-response curve for α4P451L (▼) remains monophasic and high-sensitivity activation. The curves for α4R336C (○) and α4R487Q (△) shift from a monophasic, high-sensitivity relationship to biphasic activation, with both variants gaining low-sensitivity activation components. (B) Electrophysiological recordings of $\alpha4\beta2$ nAChRs expressed in *X. laevis* oocytes activated by nicotine after 24-hour exposure to 1 μM nicotine. The concentration-response curve for the common $\alpha4$ variant (●) remains a monophasic, high-agonist sensitivity relationship, as does the curve established for α4P451L (▼). The curves for α4R336C (○) and α4R487Q (△) shift from a monophasic, high-sensitivity component to biphasic curves with added low-sensitivity components. The size of the LS component is particularly large for α4R336C.

ALS-risk variant could not be rescued by concentrations of nicotine that normally produce upregulation of nAChRs and typically observed in smoker's blood (Benowitz, 1996). Unlike the resistance of $\alpha4\beta4$ nAChRs to nicotine-initiated upregulation in vitro, we find that nicotine reliably upregulates α4P451L$\beta2$ receptors to levels consistent with the common variant of $\alpha4$. The α4R487Q variant was also identified as a risk variant for sporadic ALS (Sabatelli et al., 2012), but with lower frequency than observed for α4P451L. The results presented here demonstrate altered expression of these ALS-risk variants of the $\alpha4$ nAChR subunit and suggest that this polymorphism clearly demands additional study, both for its effects on nicotine addiction and as a contributor to cellular stress and neurodegenerative disease.

The effects of the rare *CHRNA4* variants on nAChR binding site production, plasma membrane trafficking, and upregulation by nicotine are further supported by proteomic studies. The $\alpha4$ subunit of the nAChR is phosphorylated on several sites within the M3-M4 loop (Wecker et al., 2001; Pacheco et al., 2003; Pollock et al., 2007, 2009), and we augmented the proteomic study by enriching for phosphopeptides prior to LC-MS/MS to identify and profile potential shifts in the phosphorylation state of nAChR subunits and other putatively associated proteins attributable to the presence of the $\alpha4$ rare variants. The degree of difference relative to the common variant may identify interesting targets for future mechanistic studies and is an indicator of the severity of effect of a single amino acid substitution on the function/assembly/trafficking of $\alpha4\beta2$ nAChRs conferred by these rare $\alpha4$ variants. The α4P451L and α4R336C variants have as many proteins that are unique or missing as are shared with the common $\alpha4$ variant, whereas the α4R487Q variant is less divergent and only differs by 30%. The interactome of α4P451L in particular highlights the disproportionately large effect this single amino acid substitution has on its associated proteins. The α4P451L variant binds importin subunits without any evidence of nuclear transport, fails to associate with several isoforms of 14-3-3 (a known nAChR chaperone), in the Phospho fraction, and lacks association with the transport protein VAPB in the FT fraction. This lost interaction with VAPB is important, because mutations in VAPB are strongly associated with familial ALS (Nishimura et al., 2004), adding further support to the potential involvement of the α4P451L variant in the etiology of this neurodegenerative disease.

Electrophysiological measurements of heteromeric nAChRs have demonstrated that functional properties of these receptors are strongly influenced by receptor stoichiometry (Papke et al., 1989). Experiments that alter the relative expression of individual subunits (Zwart and Vijverberg 1998; Nelson et al., 2003; Moroni and Bermudez, 2006; Gotti et al., 2008) or use concatenated $\alpha4$ and $\beta2$ subunits to force expression of defined subunit arrangements (Zhou et al., 2003), have demonstrated that functional parameters of $\alpha4\beta2$ nAChRs are largely dependent on the position of individual subunits in the pentamer. Overall, $\alpha4_2\beta2_3$ nAChRs are more sensitive to agonists than $\alpha4_3\beta2_2$ nAChRs (Nelson et al., 2003), and both stoichiometries of $\alpha4\beta2$ can be observed in the

TABLE 9
Calculated acetylcholine concentration-response parameters after 24-hour nicotine

hα4 Variant	HS EC_{50}	HS Max Response	LS EC_{50}	LS Max Response
	μM		*μM*	
Common	3.36 ± 0.40	1.05 ± 0.02	—	—
α4R336C	18.3 ± 12.3	1.05 ± 0.54	222.0 ± 65.0	3.2 ± 0.50
α4P451L	6.26 ± 0.79	1.08 ± 0.02	—	—
α4R487Q	3.5 ± 1.0	0.80 ± 0.10	128.0 ± 35.0	1.0 ± 0.09

TABLE 10
Calculated nicotine concentration-response parameters after 24-hour nicotine

hα4 Variant	HS EC_{50}	HS Max Response	LS EC_{50}	LS Max Response
	μM		μM	
Common	1.30 ± 0.36	0.28 ± 0.01	—	—
α4R336C	13.2 ± 2.20	1.90 ± 0.20	5493 ± 4.6 × 10^4	20.2 ± 158.8
α4P451L	0.97 ± 0.32	0.26 ± 0.01	—	—
α4R487Q	2.23 ± 0.50	0.29 ± 0.03	3623 ± 2.0 × 10^4	3.30 ± 17.0

mammalian CNS (Marks et al., 1999, 2007). The current studies indicate that α4 polymorphisms may shift the ratio of α4β2 nAChR assembly to favor the production of HS receptors. Consistent with this possibility, we see that ACh concentration-response curves for the common α4 variant show both HS and LS components, indicative of the presence of both α4β2 stoichiometries, whereas α4 rare variants produced monophasic, high-sensitivity ACh concentration-response curves when expressed in *X. laevis* oocytes. Both *X. laevis* oocyte and HEK 293 experiments suggest that α4P451L preferentially assumes the $\alpha4_2\beta2_3$ HS stoichiometry, likely due to decreased efficiency of α4 subunit incorporation. We did not, however, expect the same result for α4R336C and α4R487Q, which showed little difference relative to the common variant of α4 in nAChR expression or subcellular distribution when measured in transfected HEK293 cells. When expressing the receptor subunits as concatamers to restrict stoichiometry, the α4P451L variant produced HS receptors even when conditions strictly supported the assembly of LS α4β2 nAChRs, supporting the assertion that incorporation of this α4 variant is inefficient. The effects of α4R336C as a concatamer are more difficult to interpret, because both α4β2 stoichiometries were produced regardless of experiment conditions.

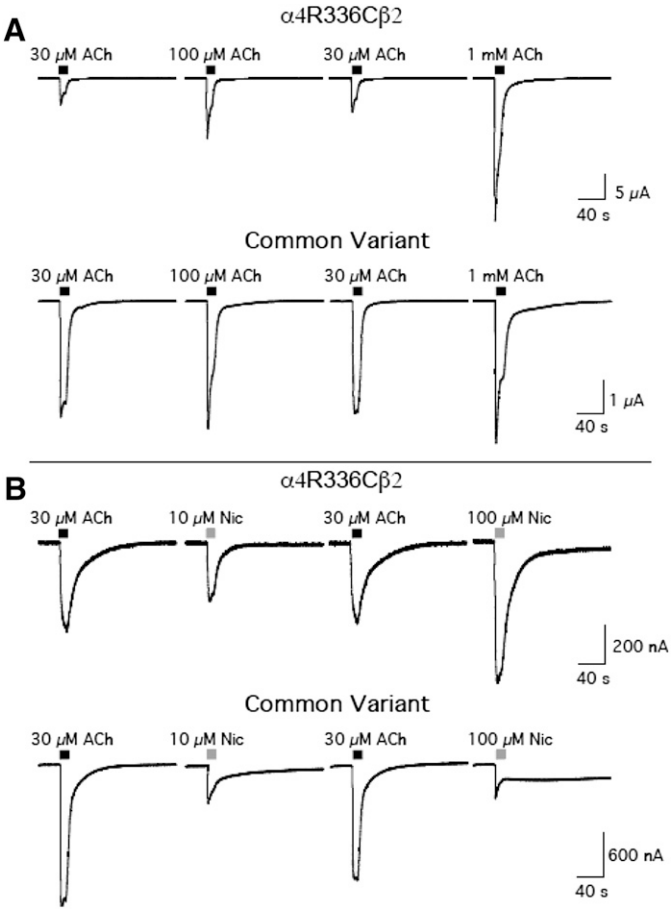

Fig. 5. Representative traces of inward currents elicited by nAChR agonists for α4R336Cβ2 and common variant α4β2 nAChRs expressed in *X. laevis* oocytes after 24-hour incubation with nicotine. (A) Inward currents elicited by acute application of ACh after 24-hour nicotine treatment. (B) Inward currents elicited by acute application of nicotine after 24-hour nicotine treatment. Both agonists reveal the presence of a distinct LS component in the α4R336C variant that is absent in oocytes injected with cRNA for the common hα4 variant after 24-hour nicotine exposure.

Upregulation of α4β2 nAChRs by nicotine exposure preferentially produces the HS form of the receptor (Kuryatov et al., 2005). After 24-hour nicotine, the common variant produced an ACh concentration-response curve with a monophasic, HS component. The α4P451L variant only produced HS nAChRs under baseline conditions and continued to produce only HS receptors after nicotine treatment. The α4R336C and α4R487Q variants, by contrast, responded to nicotine exposure by producing LS components in response to both ACh and nicotine. This effect opposes the actions of those variants on receptor assembly in the absence of nicotine. A confounding factor comes from the observation that *X. laevis* oocytes act as "nicotine sponges" during incubation with nicotine and release desensitizing concentrations of nicotine into superfusion buffer during recording (Jia et al., 2003). However, HS and LS forms of α4β2 nAChRs do not differ in their sensitivity to steady-state desensitization with subactivating concentrations of nicotine (Marks et al., 2010), so any differences in function observed after 24-hour nicotine across the α4 variants are due to effects conferred by the variants themselves.

A growing body of epidemiologic evidence suggests nicotine sensitivity is a potent determinant of smoking liability (Pomerleau et al., 2003; Hu et al., 2006). Furthermore, investigations with mouse strains led to the discovery of naturally occurring polymorphisms in nAChR subunits that influenced sensitivity to nicotine (Stitzel et al., 2000). One of these naturally occurring mouse polymorphisms was found in the intracellular loop of the α4 subunit (α4A529T) (Stitzel et al., 2001). Expression of the α4A529 variant increases sensitivity to nicotine, eliminates nicotine conditioned place preference, and produces increased HS α4β2 nAChRs in the midbrain (Wilking et al., 2010). A smoking risk-associated nAChR polymorphism that occurs in the α5 nAChR subunit (Bierut et al., 2008) introduces an amino acid substitution in the intracellular loop of α5 (α5D397N; D398N for mouse sequence) and results in decreased function of α4β2 and α3β4 nAChRs (Bierut et al., 2008; Kuryatov et al., 2011). Thus, expression of nAChRs in the CNS that favor high-agonist

sensitivity assemblies appear to be associated with a decrease in risk for nicotine dependence, whereas expression of lower-sensitivity receptors increases risk of nicotine dependence.

Conclusions

The current observations that certain rare α4 nAChR subunit variants that are apparently underrepresented among dependent smokers are more likely to assemble in the high sensitivity conformation supports the link between agonist sensitivity of neuronal nAChRs with the likelihood of nicotine dependence and addiction-related behaviors. We have shown that single amino acid substitutions in the large M3-M4 loop of the α4 nAChR subunit can result in large changes in receptor number, stoichiometry, and associated proteins. These studies also suggest potential molecular mechanisms by which these effects are produced by the individual mutations. Although our mechanistic grasp of the functional and expression differences for α4R336C and α4R487Q is not complete, changes in intracellular interactions with associated proteins or posttranslational modifications (phosphorylation, glycosylation) may be involved in the effects of these rare α4 nAChR subunit variants. For α4P451L, considerable evidence suggests that deficient assembly in the early maturational stages of the receptor results in the preferential exclusion of α4P451L from the fifth position, resulting in constrained expression of $\alpha4_2\beta2_3$ nAChRs. This mechanism is consistent with reports indicating that α4P451L is a risk variant for sporadic ALS, and such association may result from enhanced ER stress due to deficient receptor assembly.

Authorship Contributions

Participated in research design: McClure-Begley, Papke, Gelernter, Xie, Picciotto.

Conducted experiments: McClure-Begley, Papke, Stone, Stokes, Levy.

Contributed new reagents or analytic tools: Stone, Lindstrom, Xie.

Performed data analysis: McClure-Begley, Papke, Stone, Stokes, Levy.

Wrote or contributed to the writing of the manuscript: McClure-Begley, Papke, Gelernter, Picciotto.

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