

Functional Characterization of the $\alpha 5$ (Asn398) Variant Associated with Risk for Nicotine Dependence in the $\alpha 3\beta 4\alpha 5$ Nicotinic Receptor

Ping Li, Megan McCollum, John Bracamontes, Joe Henry Steinbach, and Gustav Akk

Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri

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ABSTRACT

Smoking is a major cause for premature death. Work aimed at identifying genetic factors that contribute to nicotine addiction has revealed several single nucleotide polymorphisms (SNPs) that are linked to smoking-related behaviors such as nicotine dependence and level of smoking. One of these SNPs leads to an aspartic acid-to-asparagine substitution in the nicotinic receptor $\alpha 5$ subunit at amino acid position 398 [rs16969968; $\alpha 5$ (Asn398)]. The $\alpha 5$ subunit is expressed both in the brain and in the periphery. In the brain, it associates with the $\alpha 4$ and $\beta 2$ subunits to form $\alpha 4\beta 2\alpha 5$ receptors. In the periphery, the $\alpha 5$ subunit combines with the $\alpha 3$ and $\beta 4$ subunits to form the major ganglionic postsynaptic nicotinic receptor subtype. The $\alpha 3\beta 4\alpha 5$ receptor regulates a variety of autonomic responses such as control of cardiac rate, blood pressure, and perfusion.

In this paradigm, the $\alpha 5$ (Asn398) variant may act by regulating autonomic responses that may affect nicotine intake by humans. Here, we have investigated the effect of the $\alpha 5$ (Asn398) variant on the function of the $\alpha 3\beta 4\alpha 5$ receptor. The wild-type or variant $\alpha 5$ subunits were coexpressed with the $\alpha 3$ and $\beta 4$ subunits in human embryonic kidney 293 cells. The properties of the receptors were studied using whole-cell and single-channel electrophysiology. The data indicate that the introduction of the $\alpha 5$ (Asn398) mutation has little effect on the pharmacology of receptor activation, receptor desensitization, or single-channel properties. We propose that the effect of the $\alpha 5$ (Asn398) variant on nicotine use is not mediated by an action on the physiological or pharmacological properties of the $\alpha 3\beta 4\alpha 5$ subtype.

Introduction

Smoking leads to over 400,000 premature deaths in the United States annually. More than 45 million people in the United States smoke; however, only about one third of those who initially experiment with tobacco go on to become regular smokers (McNeill, 1991). This indicates that there is significant individual variability in the progression to regular use of tobacco. Recent work attempting to determine genetic factors that contribute to nicotine addiction has identified several single-nucleotide polymorphisms (SNPs) that are associated with smoking-related behaviors such as nicotine dependence, level of smoking and age of initiation (Bierut, 2007; Bierut et al., 2007; Wang et al., 2009; Grucza et al., 2010; Saccone et al., 2010). One of these SNPs leads to an asparagine-to-aspartic acid substitution in the nicotinic

receptor $\alpha 5$ subunit at the amino acid position 398 (Saccone et al., 2007; Bierut et al., 2008). The $\alpha 5$ (Asp398) is the major allele in all populations studied, and the presence of the Asn398 variant is associated with a significantly elevated risk for increased nicotine use (Saccone et al., 2007; Bierut et al., 2008). It should be noted that the aspartic acid residue at position 398 is highly conserved across species.

The $\alpha 5$ subunit is expressed both in the brain and in the periphery. In the brain, it associates with $\alpha 4$ and $\beta 2$ subunits to form presynaptic $\alpha 4\beta 2\alpha 5$ receptors. By some accounts, up to 40% of epibatidine-labeled nicotinic receptors in the brain contain the $\alpha 5$ subunit (Brown et al., 2007; Mao et al., 2008). The $\alpha 5$ subunit is also highly expressed in the periphery, where it combines with the $\alpha 3$ and $\beta 4$ subunits to form the major postsynaptic nicotinic receptor subtype in the autonomic ganglion cells (Vernallis et al., 1993; Conroy and Berg, 1995). In the $\alpha 3\beta 4\alpha 5$ combination, the $\alpha 5$ (Asn398) variant may be involved in the regulation of autonomic responses, such as control of cardiac rate, blood pressure, and perfusion, which may affect nicotine intake in humans. In addition, the $\alpha 3$, $\beta 4$, and $\alpha 5$ subunits are expressed in a number of non-

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ABBREVIATIONS: SNP, single-nucleotide polymorphism; HEK, human embryonic kidney; DMPP, 1,1-dimethyl-4-phenylpiperazinium; NNN, *N*-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; ACh, acetylcholine.

neural cells, including bronchial and epithelial cells and lung cancer cell lines, where the activation of nicotinic receptors may play a role in tumor initiation or growth (Egleton et al., 2008).

Here, we have examined the functional effect of the $\alpha 5(\text{Asn398})$ variant on the nicotinic receptor function. The wild-type and variant $\alpha 5$ subunits were coexpressed alongside the peripheral $\alpha 3$ and $\beta 4$ subunits in HEK 293 cells and subjected to a battery of tests to investigate and compare the biophysical and pharmacological properties of wild-type and variant receptors. Our data indicate that the introduction of the $\alpha 5(\text{Asn398})$ variant has little effect on the pharmacology of the receptor, desensitization, or the major single-channel properties.

Materials and Methods

cDNAs and Molecular Biology. The experiments were conducted on human embryonic kidney (HEK) 293 cells, transiently or stably expressing combinations of human nicotinic $\alpha 3$, $\beta 4$, and $\alpha 5$ subunits. We initially created a HEK line stably expressing $\alpha 3$ and $\beta 4$ subunits. This cell line was used in transient transfections with wild-type and mutant $\alpha 5$ subunits. The cDNAs for the wild-type $\alpha 3$ (accession number NP_000734.2) and $\beta 4$ (accession number NP_000741.1) subunits were kindly provided by Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). A codon optimized human nicotinic $\alpha 5$ subunit cDNA (accession number BC033639) in pUC57 was obtained from Genscript USA Inc. (Piscataway, NJ). The cDNA was amplified by polymerase chain reaction using primers complementary to the 5' and 3' termini, introducing NotI endonuclease sites at each end of the polymerase chain reaction product. All subunits were subcloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA). The FLAG epitope [DYKDDDDK (Hopp et al., 1988)] was introduced into $\alpha 5$ between the 6 and 7 positions of the mature polypeptide using the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA). The full insert was sequenced to verify sequence integrity. The $\alpha 5\text{FLAG}$ cDNA was mutated with QuikChange to generate the $\alpha 5(\text{Asn398})$, $\alpha 5(\text{V9'S})$, and $\alpha 5(\text{Asn398}+\text{V9'S})$ mutant clones. The mutated subunits were fully sequenced to confirm that only the desired mutation(s) had been produced.

Generation of HEK 293 Cells Stably Expressing Human Nicotinic $\alpha 3\beta 4$ Receptors. HEK 293 cells (American Tissue Culture Collection, Gaithersburg, MD) were maintained in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 (1:1), also containing L-glutamine and 15 mM HEPES, with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) in a humidified atmosphere containing 5% CO_2 at 37°C. The day before transfection, the cells were plated onto two 100-mm dishes (10^6 cells per dish).

The transfection procedures have been described in detail previously (Akk, 2002). A total of 21 μg of expression constructs for the human $\alpha 3$ and $\beta 4$ subunits in the ratio of 1:1 per 100-mm dish was used in a calcium phosphate precipitation-based transfection. The cells were incubated with the precipitate at 37°C in 5% CO_2 for approximately 20 h. The transfected dishes were rinsed three times with HEK media and then returned to the incubator. Two days after transfection, transfected cells were initially selected by growth in medium containing the antibiotic G418 (1000 $\mu\text{g}/\text{ml}$; Invitrogen, Carlsbad, CA). The culture medium was changed every few days to ensure adequate cell growth.

The initially selected cells were maintained in G418, and then repeatedly immunoselected (Chen et al., 1995) with monoclonal antibody 35 (Sigma-Aldrich, St. Louis, MO), which binds to an epitope on the extracellular surface of the $\alpha 3$ subunit. We call this procedure "panning." The day before panning, 60-mm Petri dishes were coated with 3 ml of monoclonal antibody 35 at 25 $\mu\text{g}/\text{ml}$ con-

centration in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , pH 7.4). The dishes were incubated at 37°C in 5% CO_2 . On the day of panning, the cells from the 100-mm dishes were released by washing with PBS and trypsin. Five milliliters of HEK media were added to the trypsinized dish, and the cells were collected into a 15-ml centrifuge tube. The cells were then centrifuged and resuspended with 5 ml of Hanks' balanced salt solution (Mediatech, Manassas, VA) containing 5% fetal bovine serum. The entire contents of the 15-ml centrifuge tube were dispensed into the precoated Petri dish and set at room temperature for 30 min, allowing the cells to settle down and adhere to the antibody. The Petri dish was then rinsed three times with Hanks' balanced salt solution, and unattached cells were aspirated and discarded. After that, 4 ml of HEK medium containing G418 were added to the dish and the dish was returned to the incubator. Typically, it took 2 days for the immunoselected and drug-resistant cells to grow to near 100% confluence. Panning was repeated three times to establish the stable $\alpha 3\beta 4$ cell line used in this study. The cell line was then maintained in HEK growth media with 400 $\mu\text{g}/\text{ml}$ G418.

The stable $\alpha 3\beta 4$ cell line was used for transient transfections with wild-type or mutant $\alpha 5$ subunits to express $\alpha 3\beta 4\alpha 5$ receptors. Transient transfections were done using Effectene (QIAGEN, Valencia, CA) according to manufacturer's instructions. In brief, 0.4 μg of cDNA per 35-mm dish was mixed with the Enhancer and the Effectene Transfection Reagent. The cells were incubated with the mix for 6 to 18 h. Electrophysiological experiments commenced the following day.

On the day of experiments, we employed a bead-binding technique to select $\alpha 5$ expressing cells for electrophysiology (Ueno et al., 1996). The $\alpha 5$ subunits incorporated a FLAG epitope tag in the aminoterminal region. We used a mouse monoclonal antibody to the FLAG epitope (M2; Sigma-Aldrich, St. Louis, MO), which had been adsorbed to immunobeads with a covalently attached goat anti-mouse IgG antibody (Invitrogen). The cells were incubated with the beads for 5 to 10 min with gentle shaking, and cells expressing the $\alpha 5$ subunit were identified from the presence of beads bound to the cell. Expression of the subunit combinations was similar, based on the average response to 1 mM ACh: -984 ± 689 pA ($\alpha 3\beta 4$, 28 cells), -1560 ± 767 pA ($\alpha 3\beta 4\alpha 5$, 20 cells), -1981 ± 1031 pA [$\alpha 3\beta 4\alpha 5(\text{Asn398})$, 20 cells], -1502 ± 713 pA [$\alpha 3\beta 4\alpha 5(\text{V9'S})$, 20 cells], and -1431 ± 661 pA [$\alpha 3\beta 4\alpha 5(\text{Asn398} + \text{V9'S})$, 19 cells] (mean \pm S.D.). We note that all cells studied were selected because the cells bound beads.

Whole-Cell Recordings and Analyses. Macroscopic currents were recorded using whole-cell voltage clamp. The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.4. The pipet (intracellular) solution contained 140 mM CsCl, 4 mM NaCl, 4 mM MgCl_2 , 0.5 mM CaCl_2 , 5 mM EGTA, and 10 mM HEPES, pH 7.4. The drugs were applied through the bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT).

The recording and analysis of whole-cell currents have been described in detail in previous publications (Li et al., 2006). The cells were clamped at -60 mV. All experiments were carried out at room temperature (19–22°C). The current traces were low-pass filtered at 2 kHz and digitized at 10 kHz. The analysis of whole-cell currents was carried out using the pClamp 9.0 software package (Molecular Devices, Union City, CA).

Single-Channel Recordings and Analyses. Single-channel patch-clamp recordings were conducted in the cell-attached configuration. The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.4. The pipet solution contained 142 mM KCl, 5.4 mM NaCl, 1.7 mM MgCl_2 , 1.8 mM CaCl_2 , and 10 mM HEPES, pH 7.4. The agonist (ACh) was added to the pipet solution.

The recording and analysis of single-channel currents have been described in detail previously (Akk, 2001; Akk and Steinbach, 2003). The patch voltage was determined based on the combination of cell

membrane potential and the applied potential. The cell membrane potential was estimated from the reversal potential of nicotinic receptor currents, assuming that the currents reverse at 0 mV. The majority of cells had a membrane potential of -30 to -20 mV in the bath solution used. The channel activity was recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software (Molecular Devices). Before kinetic analysis, the currents were low-pass filtered at 3 to 5 kHz, and the data were idealized using the segmented- k -means algorithm (Qin et al., 1996).

Segments of high-frequency channel openings (i.e., bursts) selected by eye were isolated for further analysis. The open and closed interval durations were estimated from the idealized currents using a maximum likelihood method, which incorporates a correction for missed events (QuB Suite; <http://www.qub.buffalo.edu>). The records were initially analyzed by fitting a simple $C \leftrightarrow O$ model. The number of closed states (or open states if estimating open-time durations) was increased as long as the increase in the log-likelihood justified the addition of extra free parameters. An increase of >25 units was considered significant. The newly added states were connected to the central open state and not connected to each other. The mean interval duration was calculated as the inverse of the fitted rate constant governing the transition. The single-channel amplitudes were estimated using the Amps module in the QuB Suite, and the single-channel conductance was calculated as slope conductance using event amplitudes estimated at membrane potentials between -100 and -25 mV.

Reagents and Statistical Tests. The reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Tocris Bioscience (Ellisville, MO). Stock solutions (100–500 mM) of ACh, nicotine, cytosine, or 1,1-dimethyl-4-phenylpiperazinium (DMPP) were made in distilled water. Final dilutions to the bath or pipet solutions were made as needed on the day of the experiment.

Statistical analyses were carried out using a paired t test (Excel, Microsoft, Richmond, WA). Data are reported as mean \pm S.D. The fits of averaged concentration-effect curves and exponential curves were conducted using NFIT (The University of Texas, Medical Branch at Galveston, Galveston, TX), and results are reported as best-fitting value \pm estimated 95% interval of the value.

Results

Macroscopic Pharmacological Profiles of $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ Receptors. We determined the pharmacological profiles for the $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5$ (Asn398) receptors. The receptors were expressed in HEK 293 cells. The cells were exposed to 1 to 10,000 μ M ACh, 1 to 300 μ M nicotine, 1 to 300 μ M cytosine, or 1 to 300 μ M DMPP. In the cases of nicotine, cytosine, and DMPP, the responses were normalized to the peak response elicited by 1 mM ACh from the same cell. Our initial data suggested that there was relatively little difference in responses of receptors containing $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ subunits, which led to a concern that the $\alpha 5$ subunit was poorly incorporated into functional surface receptors. Accordingly, we inserted a FLAG epitope near the amino terminus of the $\alpha 5$ subunit (see *Materials and Methods*), and selected cells for study that bound small beads coated with antibody to the FLAG epitope. This approach meant that only cells expressing the $\alpha 5$ subunit were studied. To confirm that the $\alpha 5$ subunit was present in the receptors that responded to agonists, we also used a “reporter” mutation (a mutation of the 9' position in the second transmembrane region), which produces a characteristic increase in the potency of agonists (i.e., a shift of concentration-response

relationship to lower concentration of agonist) (Labarca et al., 1995; Krashia et al., 2010).

The data demonstrate that ACh and nicotine have higher efficacy on any of the subunit combinations than cytosine or DMPP, for which the peak responses range from 50 to 80% of the ACh response. The EC_{50} for $\alpha 3\beta 4$ receptors activated by ACh was 142 μ M, similar to the values observed previously (Gerzanich et al., 1998; Krashia et al., 2010). The addition of the $\alpha 5$ subunit resulted in channels with a slightly reduced EC_{50} (119 μ M). A similar leftward shift was previously observed for $\alpha 3\beta 4\alpha 5$ receptors expressed in oocytes (Gerzanich et al., 1998). The incorporation of the $\alpha 5$ (Asn398) variant further left-shifted the ACh concentration-response curve ($EC_{50} = 57 \mu$ M). However, when the midpoints of concentration-response curves from individual cells were compared, the $\alpha 5$ (Asn398) variant was not statistically different from the $\alpha 3\beta 4\alpha 5$ (not shown). The introduction of the V9'S mutation to the second membrane-spanning domain strongly left-shifted the concentration-response curves. The ACh EC_{50} values were 16 or 19 μ M when the V9'S mutation was introduced onto the $\alpha 5$ (Asp398) or $\alpha 5$ (Asn398) background, respectively. In each case the effect of the V9'S mutation was statistically significant ($p < 0.03$, two-tailed t test). The leftward shift in the concentration-response curves is expected given the nature and location of the mutation (Krashia et al., 2010) and serves as proof for the presence of the $\alpha 5$ subunit in the functional receptors.

The EC_{50} values for nicotine were tightly grouped (14–23 μ M) for the $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5$ (Asn398) receptors. The introduction of the V9'S mutation to the $\alpha 5$ subunit left-shifted the midpoints of the concentration-response curves by 3- to 8-fold. Activation by cytosine resulted in peak currents from $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5$ (Asn398) receptors that were 53 to 65% of the response to 1 mM ACh. The estimated EC_{50} values were 15 to 21 μ M. The presence of the gain-of-function V9'S mutation enhanced the relative cytosine response to 73 to 76% and shifted the concentration-response curves to lower cytosine concentrations. Receptors activated by DMPP showed peak currents of 60 to 80% of that in the presence of ACh. The midpoints of the concentration-response curves were 8 to 16 μ M for the $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5$ (Asn398) receptors and 2 to 5 μ M for the receptors containing the $\alpha 5$ (V9'S) mutation. The DMPP concentration-response curves suggested that the presence of the $\alpha 5$ (Asn398) variant may enhance the maximal current (Fig. 1D; Table 1) compared with $\alpha 5$ (Asp398). To verify this, we separately compared responses to 1 mM ACh and 100 μ M DMPP from the same set of cells. The relative response to DMPP was $71 \pm 5\%$ ($n = 5$ cells) and $67 \pm 7\%$ ($n = 6$ cells) in cells expressing $\alpha 3\beta 4\alpha 5$ and $\alpha 3\beta 4\alpha 5$ (Asn398) receptors, respectively. We conclude that the $\alpha 5$ (Asn398) variant does not modulate receptor sensitivity to DMPP.

The concentration-response data are summarized in Table 1 and the concentration-response curves for $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5$ (Asn398) receptors are shown in Fig. 1. Our overall conclusion is that the presence of the $\alpha 5$ (Asn398) variant has little effect on the agonist concentration-response properties, compared with the major $\alpha 5$ (Asp398) allele.

We also tested receptor activation by epibatidine. It is an extremely potent agonist, producing responses at concentrations as low as 10 nM on some types of nicotinic receptors. In our hands, exposure to epibatidine resulted in a long-lasting

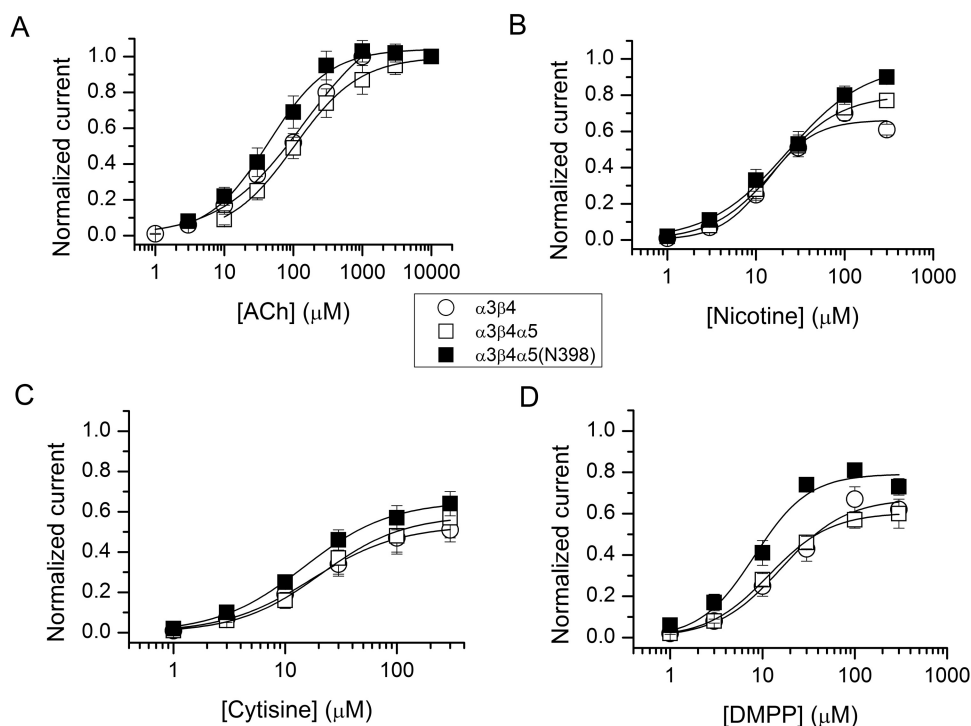


Fig. 1. Summary of activation concentration-response curves. The plots show concentration-response curves for ACh (A), nicotine (B), cytosine (C), and DMPP (D) for HEK cells stably expressing human $\alpha 3\beta 4$ receptors or stable $\alpha 3\beta 4$ cells transiently transfected with wild-type or variant (Asn398) $\alpha 5$ subunits. The $\alpha 5$ subunits contained a FLAG epitope inserted to the amino terminus of the subunit. Cells expressing the $\alpha 5$ subunit were selected with immunobeads coated with anti-FLAG antibody. Each data point represents mean \pm S.E.M. from four to seven cells. The data were normalized to the highest ACh concentration used (A) or to the 1 mM ACh response obtained from the same cell (B–D). The curves were fitted with the Hill equation. The fitting results are given in Table 1.

TABLE 1

Activation properties of the human $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5^*$ receptors

The table gives the concentration-response data for the $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, $\alpha 3\beta 4\alpha 5(\text{N398})$, $\alpha 3\beta 4\alpha 5(\text{V9'S})$, and $\alpha 3\beta 4\alpha 5(\text{Asn398} + \text{V9'S})$ receptors. The parameters are estimated by fitting data from n cells with the Hill equation, and the mean \pm S.D. parameter values are given. All data were first normalized to the response to 1 mM ACh from the same cell. I_{max} gives the fit relative maximal response, EC_{50} is the concentration producing a half-maximal response, and n_H is the Hill coefficient for the fit. Fig. 1 shows the averaged concentration response relationships for the cells.

Agonist and Parameter	$\alpha 3\beta 4$	$\alpha 3\beta 4\alpha 5$	$\alpha 3\beta 4\alpha 5(\text{Asn398})$	$\alpha 3\beta 4\alpha 5(\text{V9'S})$	$\alpha 3\beta 4\alpha 5(\text{Asn398} + \text{V9'S})$
ACh					
I_{max}	1.17 ± 0.14	1.16 ± 0.15	1.06 ± 0.06	1.09 ± 0.02	1.08 ± 0.04
EC_{50} , μM	142 ± 103	119 ± 68	57 ± 23	16 ± 10	19 ± 14
n_H	0.98 ± 0.30	1.02 ± 0.30	0.95 ± 0.20	0.82 ± 0.31	0.77 ± 0.20
n	8	5	5	5	4
Nicotine					
I_{max}	0.67 ± 0.08	0.81 ± 0.09	0.95 ± 0.10	0.88 ± 0.07	0.91 ± 0.31
EC_{50} , μM	14 ± 4	20 ± 10	23 ± 12	2 ± 1	9 ± 5
n_H	1.66 ± 0.33	1.36 ± 0.26	1.12 ± 0.17	1.45 ± 0.75	1.15 ± 0.30
n	6	5	5	4	5
Cytisine					
I_{max}	0.54 ± 0.15	0.58 ± 0.20	0.65 ± 0.13	0.77 ± 0.06	0.82 ± 0.05
EC_{50} , μM	21 ± 12	25 ± 14	15 ± 4	1 ± 1	4 ± 3
n_H	1.17 ± 0.15	1.27 ± 0.34	1.16 ± 0.18	0.92 ± 0.34	1.03 ± 0.27
n	5	5	5	5	4
DMPP					
I_{max}	0.67 ± 0.13	0.61 ± 0.13	0.79 ± 0.05	0.81 ± 0.13	0.91 ± 0.17
EC_{50} , μM	18 ± 8	12 ± 3	7 ± 1	6 ± 2	3 ± 2
n_H	1.40 ± 0.17	1.35 ± 0.18	1.39 ± 0.18	1.03 ± 0.47	0.76 ± 0.08
n	5	5	3	5	4

loss of response, especially at high concentrations. However, there seemed to be no major effects on receptors including either variant of the $\alpha 5$ subunit. We compared the responses of cells to 1 mM ACh and a single application of 1 μM epibatidine. The relative responses were 1.2 ± 0.2 ($n = 7$ cells), 1.1 ± 0.1 (5 cells), and 1.3 ± 0.3 (5 cells) for responses from $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5(\text{Asn398})$ receptors, respectively. Accordingly, it does not seem that inclusion of the $\alpha 5(\text{Asn398})$ variant had a significant effect on responses to epibatidine.

We also examined receptor activation by cotinine, *N*-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Cotinine is a major metabolite

of nicotine, which has a long half-life in the body, whereas its nominal concentration can be much higher than that of nicotine. Its nicotine-mimicking effects are likely mediated by its ability to activate the nicotinic receptor, albeit with potency that is less than nicotine's by 2 orders of magnitude (O'Leary et al., 2008). NNN and NNK are potent carcinogens found in tobacco products, which can interact with the nicotinic receptor (Schuller, 2007).

All three drugs were ineffective at direct activation, with peak currents $<1\%$ of the response to saturating ACh. No differences were observed for the $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, or $\alpha 3\beta 4\alpha 5(\text{Asn398})$ receptors. We examined the modulatory effects of the drugs by coapplying NNN or NNK at concentra-

tions up to 100 μM , or cotinine at concentrations up to 1 mM with 30 μM ACh ($\sim\text{EC}_{20}$). Overall, the data indicate that the drugs are weak inhibitors of the nicotinic receptor. The effects in the presence of NNN or NNK were minor, although in some cases statistically significant. For cotinine, we observed a strong and significant effect at 1 mM, whereas lower concentrations were largely without effect. The data are summarized in Table 2. As a positive control, we tested the effect of 5 mM tetraethylammonium, a low-efficacy agonist of the nicotinic receptor (Akk and Steinbach, 2003). Coapplication of tetraethylammonium with 30 μM ACh reduced the peak response from cells expressing $\alpha 3\beta 4$ receptors to $3 \pm 4\%$ of control ($n = 4$ cells; $p < 0.001$).

Desensitization Properties of $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5(\text{Asn}398)$ Receptors. The desensitization properties of the receptors were examined by employing a 30-s exposure to 1 mM ACh or 100 μM nicotine (both are near-saturating concentrations). The extent of desensitization showed variability and the data traces themselves were in several cases not amenable to fits by exponential components. Accordingly, we analyzed the data with regard to the time for decay from peak to 50% of peak for ACh or 80% for nicotine and the extent of residual current at the end of the 30-s drug application. The time to 80% of peak was chosen for analysis of data with nicotine, because the decay was markedly slowed by the presence of either $\alpha 5$ subunit, and the current did not always decay to 50% of peak during the 30-s application.

A previous study (Ifune and Steinbach, 1993) examining the properties of ACh-activated currents in rat pheochromocytoma cells found that the rate of desensitization was highly dependent on recording time. As the time spent in whole-cell configuration increased the peak current and rate of desensitization were enhanced. Here, we did not see consistent changes in peak amplitude. However, the desensitization rate increased with recording time (data not shown). Accordingly, to minimize the effects from intrinsic changes in desensitization, the analysis was conducted on traces that were started within the first 30 s of entering the whole-cell mode.

The 100-to-50% decay time was 15 s in $\alpha 3\beta 4$ receptors activated by 1 mM ACh (Table 3). The inclusion of the $\alpha 5$ or $\alpha 5(\text{Asn}398)$ subunit had no significant effect on desensitization in the presence of ACh. The 100-to-50% decay times ranged from 13 to 17 s for the $\alpha 3\beta 4\alpha 5$ and $\alpha 3\beta 4\alpha 5(\text{Asn}398)$ receptors. The residual current in the end of the 30-s drug application was similar at 25 to 29% of peak for the three-subunit combinations. We infer from the data that the pres-

TABLE 3

The effect of the $\alpha 5$ subunit on receptor desensitization

The receptors were activated by 1 mM ACh or 100 μM nicotine. The desensitization time courses were examined with respect to 100 to 50% of peak (or 80% for nicotine) decay time and the residual current levels at the end of the 30-s application. The data are mean \pm S.D. from five to eight cells. All estimates are from traces started within 30 s of entering the whole-cell configuration. The statistical analysis applies to comparison with the data from the $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ receptors.

Agonist and Receptor	Decay Time		Residual Current
	100-to-50%	100-to-80%	
	<i>s</i>		<i>%</i>
ACh			
$\alpha 3\beta 4$	15 \pm 12		29 \pm 23
$\alpha 3\beta 4\alpha 5$	13 \pm 6 [†]		25 \pm 14 ^{†,‡}
$\alpha 3\beta 4\alpha 5(\text{Asn}398)$	17 \pm 3 ^{†,‡}		29 \pm 7 [†]
Nicotine			
$\alpha 3\beta 4$		0.9 \pm 0.6	9 \pm 3
$\alpha 3\beta 4\alpha 5$		8.6 \pm 6.2*	50 \pm 25**
$\alpha 3\beta 4\alpha 5(\text{Asn}398)$		9.6 \pm 3.7***,†	48 \pm 15***,†

[†] Not significant.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

ence of the $\alpha 5$ subunit, wild-type, or containing the $\alpha 5(\text{Asn}398)$ variant does not influence receptor desensitization in the presence of ACh.

The rates of desensitization in the presence of 100 μM nicotine depended on the presence of the $\alpha 5$ subunit. Cells expressing $\alpha 3\beta 4$ receptors had a 100-to-80% decay time of 0.9 s. When the $\alpha 5$ subunit was included in receptor complexes, the decay time increased to 8.6 s. The introduction of the $\alpha 5(\text{Asn}398)$ variant did not further influence receptor desensitization ($\tau = 9.6$ s). Sample current traces are shown in Fig. 2, and the summary of the analysis, including statistical significance data, are given in Table 3.

We next examined the time courses for recovery from desensitization. Receptor desensitization was induced by prolonged (30 s) applications of 1 mM ACh or 100 μM nicotine. The desensitizing pulse was followed (1–120 s later) with a brief pulse whose peak response was compared with that of the control application (Fig. 3). For the majority of receptor-agonist combinations, best fits for the time courses contained two exponential components. For the $\alpha 3\beta 4$ receptor, the two components had mean durations of approximately 5 and 89 s in the presence of ACh. The components had roughly equal amplitudes. Receptors exposed to nicotine exhibited time courses with mean durations of the components at 4 and 55 s. The addition of the $\alpha 5$ subunit, and the introduction of the $\alpha 5(\text{Asn}398)$ variant had a relatively small effect on the recovery time course for desensitization induced by ACh (Table 4). It is noteworthy that the recovery time course for $\alpha 3\beta 4\alpha 5(\text{Asn}398)$ receptors in the presence of nicotine contained a single component, which had a time constant that was intermediate to the fast and slow components seen with $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ receptors and similar to the weighted time constant from these receptors.

To gain more insight into the effect of the $\alpha 5$ subunit on recovery, we compared fractional recovery after a 30-s wash-out. After desensitization by 1 mM ACh, the mean fractional recovery was $79 \pm 15\%$ ($n = 6$ cells), $79 \pm 11\%$ ($n = 5$), and $69 \pm 13\%$ ($n = 5$) for cells expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5(\text{Asn}398)$ receptors. The effect of the $\alpha 5(\text{Asn}398)$ variant was statistically insignificant. After 100 μM nicotine, the $\alpha 3\beta 4$ receptors recovered to $65 \pm 6\%$ ($n = 6$ cells)

TABLE 2

Inhibition by NNN, NNK, and cotinine

The table summarizes the modulatory effects of nicotine metabolites NNN, NNK, and cotinine. NNN (100 μM), NNK (100 μM), or cotinine (1 mM) were coapplied with ACh (at EC_{20}). The values are mean \pm S.D. of control currents from five to eight cells for each condition. The statistical significance applies to comparison with control currents.

Receptor	NNN	NNK	cotinine
	<i>%</i>		
$\alpha 3\beta 4$	92 \pm 10 [†]	92 \pm 12 [†]	53 \pm 9***
$\alpha 3\beta 4\alpha 5$	96 \pm 11 [†]	96 \pm 4 [†]	60 \pm 10***
$\alpha 3\beta 4\alpha 5(\text{Asn}398)$	88 \pm 5**	95 \pm 2**	52 \pm 12***

[†] Not significant.

** $P < 0.01$.

*** $P < 0.001$.

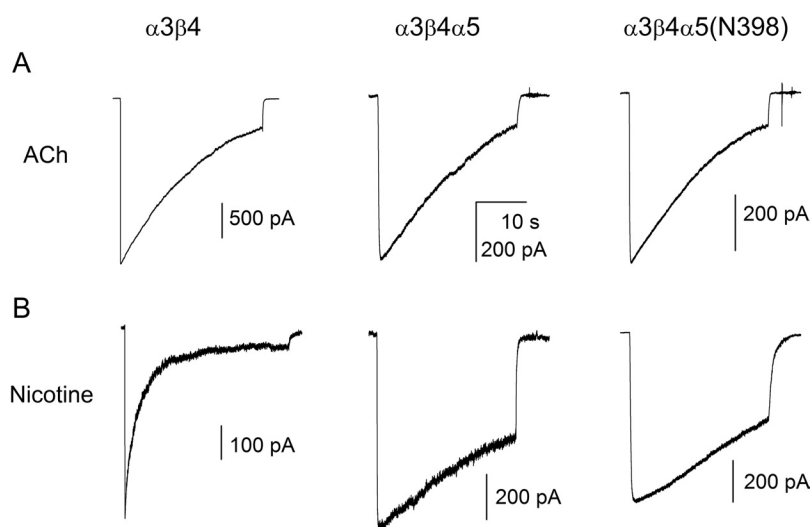


Fig. 2. Receptor desensitization in the presence of ACh or nicotine. The cells were exposed to 30 s pulses of 1 mM ACh (A) or 100 μM nicotine (B). The inclusion of the $\alpha 5$ subunit reduced the rate of desensitization in the presence of nicotine but was without effect when the receptors were activated by ACh. The summary of the analysis is given in Table 3.

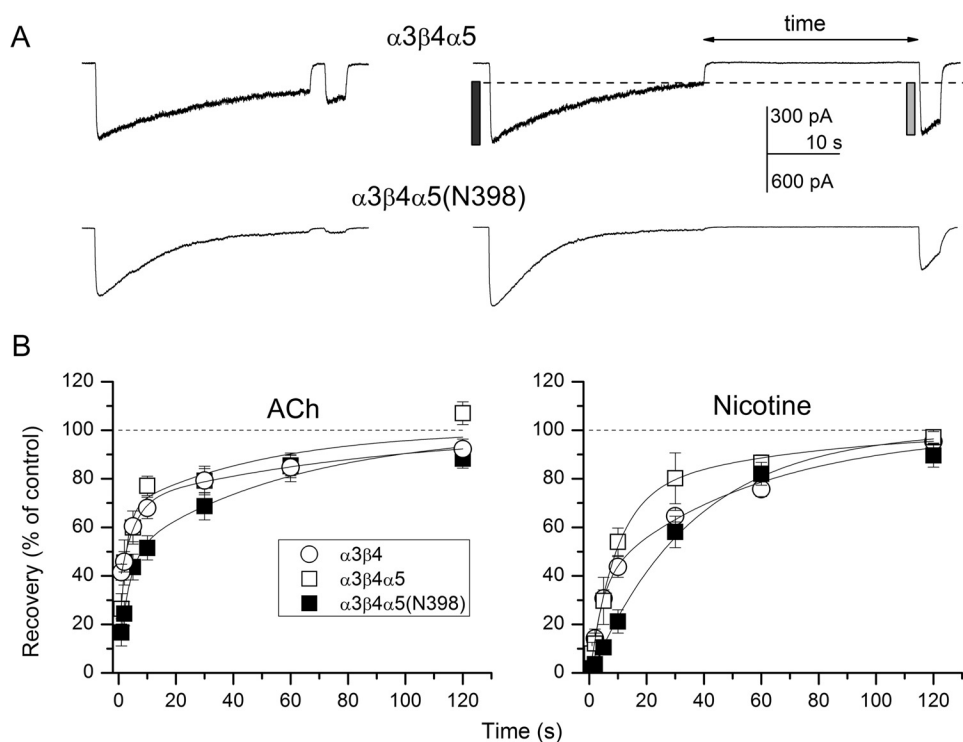


Fig. 3. Recovery from desensitization. Desensitization was induced by 30-s exposure to 1 mM ACh or 100 μM nicotine. Recovery was measured with brief pulses of the same agonist after washout in bath solution for 1 to 120 s. A, sample recordings from cells expressing $\alpha 3\beta 4\alpha 5$ (top) or $\alpha 3\beta 4\alpha 5(\text{Asn398})$ (bottom) receptors. The two traces show recovery after washout for 2 s (left) or 30 s (right). Recovery was calculated as the ratio of second peak minus residual current (gray bar in the top right trace) over control peak minus residual current (black bar). Note that the extent of desensitization is greater than that shown in traces in Fig. 2. This is so because the rate and extent of desensitization correlated with recording time. B, recovery time courses were measured for $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5(\text{Asn398})$ receptors. The curves were fitted to a single exponential [$\alpha 3\beta 4\alpha 5(\text{Asn398})$ + nicotine] or the sums of two exponentials (everything else). The fitting results are given in Table 4.

of control after a 30-s washout. Recovery was $80 \pm 18\%$ ($n = 3$) for $\alpha 3\beta 4\alpha 5$ receptors and $58 \pm 13\%$ ($n = 4$) for $\alpha 3\beta 4\alpha 5(\text{Asn398})$ receptors. The differences were not statistically significant, indicating that the presence of a single component in the recovery time course from $\alpha 3\beta 4\alpha 5(\text{Asn398})$ receptors does not result in significant changes in recovery from desensitization.

Single-Channel Currents from $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5^*$ Receptors. We investigated the effect of subunit composition and the $\alpha 5(\text{V9'S})$ mutation on single-channel parameters. The $\alpha 3\beta 4$ stable cell line was supertransfected with the $\alpha 5$, $\alpha 5(\text{Asn398})$, $\alpha 5(\text{V9'S})$, or $\alpha 5(\text{Asn398} + \text{V9'S})$ subunit. The currents were recorded in the cell-attached configuration.

Single-channel activity elicited by 3 μM ACh consisted of a mixture of bursts of openings interlaced with single openings. The majority of single-channel data fell to a single conductance class (32–34 pS; see below), but in some cells, an

additional, higher (> 50 pS) conductance class was observed. Because of its relative scarcity, the higher conductance class was not characterized.

Sample recordings are shown in Fig. 4. The intraburst open time histograms were adequately fitted to a single exponential. The $\alpha 3\beta 4$ receptors had a mean open duration of 10.7 ± 4.0 ms ($n = 4$ patches). In $\alpha 3\beta 4\alpha 5$ receptors, the mean open duration was 3.5 ± 1.0 ms ($n = 3$ patches). As expected, the introduction of the $\alpha 5(\text{V9'S})$ mutation prolonged channel openings, increasing the mean open duration to 9.7 ± 1.8 ms ($n = 3$ patches). The presence of the $\alpha 5(\text{Asn398})$ variant had no significant effect on the mean open time (4.7 ± 0.9 ms; $n = 3$ patches) compared with $\alpha 3\beta 4\alpha 5$. In the receptor containing the $\alpha 5(\text{Asn398} + \text{V9'S})$ double mutation, the mean open time was 9.7 ± 1.4 ms ($n = 4$ patches). We conclude that the $\alpha 5(\text{Asn398})$ variant is without effect on the open-time durations, regardless of the presence of the background $\alpha 5(\text{V9'S})$

TABLE 4
The effect of the $\alpha 5$ subunit on recovery from desensitization
The receptors were exposed to 30-s pulses of 1 mM ACh or 100 μ M nicotine, eliciting channel desensitization. Recovery from desensitization was tested with brief (3-s) pulses at 1- to 120-s time points. The table gives the fitting results (best fit estimates and 95% confidence limits) for curves describing the averaged recovery data points from four to eight cells.

Receptor	$\tau 1$ (fraction)	$\tau 2$
	s (%)	s
ACh		
$\alpha 3\beta 4$	4.8 ± 0.8 (55)	89 ± 13
$\alpha 3\beta 4\alpha 5$	2.2 ± 1.8 (66)	49 ± 32
$\alpha 3\beta 4\alpha 5$ (Asn398)	3.5 ± 2.0 (45)	59 ± 16
Nicotine		
$\alpha 3\beta 4$	3.9 ± 1.4 (40)	55 ± 8
$\alpha 3\beta 4\alpha 5$	8.8 ± 1.9 (73)	66 ± 31
$\alpha 3\beta 4\alpha 5$ (Asn398)	36 ± 3 (100)	N/A

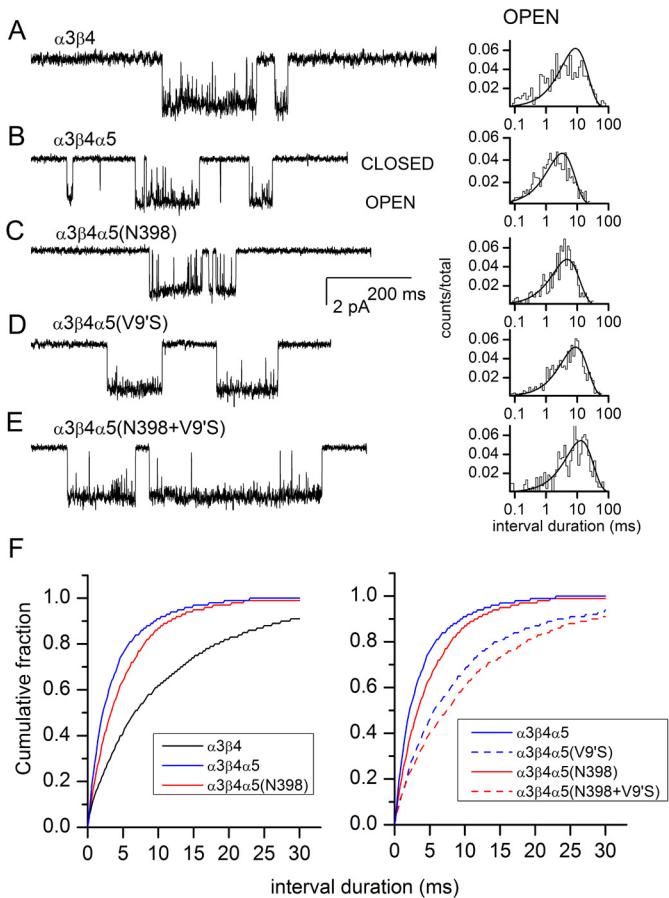


Fig. 4. Single-channel currents from the wild-type and mutant receptors activated by 3 μ M ACh. A, the currents were recorded from a HEK cell stably expressing the human $\alpha 3\beta 4$ receptors. The stably expressing $\alpha 3\beta 4$ cells were used in transient transfections with wild-type (B) and mutant (C–E) $\alpha 5$ subunits. The wild-type and mutant $\alpha 5$ subunits contained a FLAG epitope inserted at the amino terminus of the subunit. Cells expressing the $\alpha 5$ subunit were selected with immunobeads coated with anti-FLAG antibody. The currents were recorded in the cell-attached configuration. Channel openings are shown as downward deflections. The intraburst open times were fitted with a single exponential. The mean open times were 13.3 ms (A), 2.7 ms (B), 4.3 ms (C), 7.1 ms (D), and 9.7 ms (E). The average open times from multiple patches are given in the text. The intraburst closed-time durations are given in Table 3. F, cumulative open-time distribution histograms. The addition of the $\alpha 5$ subunit results in briefer open-time durations (left). The introduction of the V9'S mutation to the $\alpha 5$ subunit results in longer open time durations (right).

mutation. Conversely, the data also show that the V9'S mutation similarly affects the open durations from receptors containing both variants of the $\alpha 5$ subunit. Cumulative plots of the open durations are shown in Fig. 4F. The reduction in open time after introduction of $\alpha 5$ or $\alpha 5$ (Asn398) is highly significant ($p < 0.001$ for comparison to $\alpha 3\beta 4$; Kolmogorov-Smirnov 2-tailed test). The differences between $\alpha 5$ and $\alpha 5$ (V9'S) and between $\alpha 5$ (Asn398) and $\alpha 3\beta 4\alpha 5$ (Asn398+V9'S) are also highly significant ($p < 0.001$ for each comparison).

The intraburst closed times contained two components. The briefer component had a mean duration of approximately 0.1 ms, making up the majority (90%) of all intraburst closed intervals. The longer-lived component had a mean duration of 1 to 3 ms. The presence of the $\alpha 5$ subunit did not affect the closed time distributions. The closed time data are summarized in Table 5.

We determined the slope conductance of single-channel activity by measuring the current-voltage relationship at membrane potentials of -100 to -25 mV. The single-channel conductances were indistinguishable: $\alpha 3\beta 4$ receptors, 34.0 ± 2.1 pS ($n = 4$ patches); $\alpha 3\beta 4\alpha 5$, 33.7 ± 3.6 pS ($n = 3$ cells); and $\alpha 3\beta 4\alpha 5$ (Asn398), 32.8 ± 1.9 pS ($n = 4$ cells).

A previous study employing outside-out patches from *Xenopus laevis* oocytes expressing $\alpha 3\beta 4$ or $\alpha 3\beta 4\alpha 5$ receptors found a single-channel conductance of 31 and 36 pS, respectively (Nelson and Lindstrom, 1999). Nicotinic currents from rat superior cervical ganglion cells, where $\alpha 3\beta 4$ receptors form a major nicotinic receptor component, show a single-channel conductance of 35 to 37 pS (Sivilotti et al., 1997). Rat $\alpha 3\beta 4$ receptors expressed in BOSC 23 cells predominantly shows a 34-pS conductance class (Ragozzino et al., 1997). Overall, our findings are similar to the previously published single-channel data on wild-type $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ receptors (e.g., Lewis et al., 1997; Nelson and Lindstrom, 1999; Boorman et al., 2003). Our single-channel data indicate that the $\alpha 5$ (Asp398) and $\alpha 5$ (Asn398) variants behave similarly in single-channel recordings.

Discussion

The neuronal nicotinic $\alpha 5$ subunit does not express as a homo-oligomer, and it cannot substitute for other neuronal α subunits that combine with a β subunit to form functional channels. Rather, the $\alpha 5$ subunit acts as an accessory subunit combining with, and in some cases modulating, other $\alpha\beta$ hetero-oligomeric receptors. The $\alpha 5$ subunit is expressed in the brain, where it contributes to the $\alpha 4\beta 2\alpha 5$ receptor, and in the periphery, where it combines with $\alpha 3$ and $\beta 4$ subunits to form the major ganglionic postsynaptic receptor (Vernallis et

TABLE 5
The effect of the $\alpha 5^*$ subunit on the intraburst closed-time properties
The intraburst activity was fitted to the sums of two exponentials. The data are mean \pm S.D. from three to four patches. The columns give receptor subunit composition and the mean durations and fractions of the two closed-time components.

Receptor	CT1	Fraction CT1	CT2	Fraction CT2
	ms		ms	
$\alpha 3\beta 4$	0.11 ± 0.05	0.88 ± 0.05	1.6 ± 0.5	0.12 ± 0.05
$\alpha 3\beta 4\alpha 5$	0.11 ± 0.03	0.91 ± 0.06	0.8 ± 0.3	0.09 ± 0.06
$\alpha 3\beta 4\alpha 5$ (Asn398)	0.12 ± 0.04	0.80 ± 0.18	0.8 ± 0.4	0.20 ± 0.18
$\alpha 3\beta 4\alpha 5$ (V9'S)	0.10 ± 0.02	0.88 ± 0.07	2.6 ± 1.7	0.12 ± 0.07
$\alpha 3\beta 4\alpha 5$ (Asn398 + V9'S)	0.10 ± 0.01	0.96 ± 0.001	1.2 ± 0.6	0.04 ± 0.001

al., 1993; Conroy and Berg, 1995). In addition, the nicotinic $\alpha 5$ subunit is expressed in lung epithelial cells with $\alpha 3$ and $\beta 4$ and in small-cell and non-small-cell lung cancer cell lines (Maus et al., 1998; Egletton et al., 2008; Song and Spindel, 2008). We examined the effects of including two sequence variants of the $\alpha 5$ subunit on the physiological and pharmacological properties of $\alpha 3\beta 4$ receptors. The minor allele [$\alpha 5(\text{Asn398})$] is associated with increased risk of developing nicotine dependence (Saccone et al., 2007; Bierut et al., 2008). We considered the possibility that an effect on the function of ganglionic neuronal nicotinic receptors might significantly alter the actions of nicotine to produce aversive, or preferred, peripheral responses, and so modify behavioral responses.

We employed whole-cell and single-channel patch clamp to investigate the physiological and pharmacological properties of $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 4\alpha 5(\text{Asn398})$ receptors. We also generated and studied receptors containing a valine-to-serine mutation in the second membrane-spanning domain (V9'S) of the $\alpha 5$ subunit. This gain-of-function mutation acted as a functional reporter for the expression and incorporation of the $\alpha 5$ subunit.

Our findings indicate that the $\alpha 5(\text{Asn398})$ variant has no functional effect in the $\alpha 3\beta 4\alpha 5$ receptor compared with the major allele, insofar as covered by the tests that we employed. Specifically, the variant was without effect on channel activation by ACh, nicotine, cytosine or DMPP, or the nicotine metabolites cotinine, NNN, or NNK. The variant was also without effect on channel desensitization properties, or single-channel parameters such as intracurrent open and closed times and single-channel conductance.

Given its passive nature, the expression and incorporation of the $\alpha 5$ subunit cannot be determined simply from the observation of a functional response. Nor are we aware of any specific antibodies to the $\alpha 5$ subunit or a pharmacological approach to eliminate non- $\alpha 5$ containing receptors. Accordingly, we engineered a FLAG epitope tag at the amino terminus of the subunit. Cells expressing the $\alpha 5$ subunit on the surface were selected by beadbinding. Although this approach does not guarantee the presence of the $\alpha 5$ subunit in each receptor-complex, bead binding indicates the incorporation of the $\alpha 5$ subunit in, at least, some surface receptors. The presence of the $\alpha 5$ subunit in the functional receptors we studied is confirmed by the leftward shift on the agonist concentration-response curves and the related prolongation of open durations observed when the $\alpha 5$ subunit contained the V9'S mutation in the second membrane-spanning domain. Original studies on the related muscle-type nicotinic receptor have shown that mutations that enhance the polarity of the 9' residue (a leucine in most subunits) facilitate channel opening by reducing the channel-closing rate constant (Filatov and White, 1995; Labarca et al., 1995; Chen and Auerbach, 1998). This results in a leftward shift in the agonist concentration-response curve. Accordingly, we used this approach to probe for the presence of the $\alpha 5$ subunit. Our data indicate that the introduction of the V9'S mutation in the $\alpha 5$ subunit prolongs the mean open duration in single-channel recordings (Fig. 4) and shifts the macroscopic concentration-response curves to lower agonist concentrations (Table 1). It is noteworthy that the presence of the Asn398 variant on the $\alpha 5(\text{V9'S})$ background did not influence macroscopic parameters or the properties of single-channel cur-

rents, compared with the major allele. These findings are important, because in these experiments, the presence of the $\alpha 5$ subunit in receptor-complexes is unequivocally shown by V9'S-specific changes in channel properties.

It should be mentioned that a previous study examining currents from $\alpha 3\beta 4\alpha 5$ receptors expressed in *X. laevis* oocytes found that a valine-to-threonine mutation at the 9' position was without effect on the ACh concentration-response curve. The only reported effect of the mutation was a reduction in the apparent rate of desensitization (Groot-Kormelink et al., 2001).

A recent study demonstrated that $\alpha 3\beta 4$ receptors exist in two stoichiometries: one with two $\alpha 3$ subunits and three $\beta 4$ subunits, the other containing three $\alpha 3$ s and two $\beta 4$ s (Krashia et al., 2010). The two stoichiometric isoforms differed in their relative sensitivity to DMPP (the three α isoform was more sensitive) and single-channel properties (the three α isoform had a larger single-channel conductance). Examination of our data suggests that the stable $\alpha 3\beta 4$ cell line used in this study expresses receptors in the 3:2 ($\alpha 3:\beta 4$) stoichiometry. The ratio of EC_{50} values for ACh versus DMPP is 7.7 (Table 1), similar to the 10-fold difference found by Krashia et al. (2010) for the three α isoform. However, our single-channel conductance estimate for $\alpha 3\beta 4$ receptors (34 pS) is intermediate to the values for the two isoforms (26 and 39 pS for 2:3 and 3:2, respectively). It is not clear whether the discrepancy results from differences in recording conditions (cell-attached versus outside-out) or indicates that our cells express both isoforms.

Our estimates for activation properties of the $\alpha 3\beta 4$ receptor show similarities as well as differences with previous studies. For example, our estimate for the EC_{50} for DMPP (16 μM) is close to the values obtained previously (10–18 μM ; Gerzanich et al., 1998; Stauderman et al., 1998). Likewise, our estimate for the EC_{50} for ACh (142 μM) agrees with Gerzanich et al. (1998) (163 μM) and Papke et al. (2010) (79 μM). On the other hand, the estimates for nicotine potency vary considerably, with EC_{50} values at 14 μM (Table 1), 40 μM (Stauderman et al., 1998), or 106 μM (Gerzanich et al., 1998). A study examining stimulation of $^{86}\text{Rb}^+$ flux yielded EC_{50} values of 28 and 114 μM for nicotine and ACh, respectively (Xiao et al., 1998). Our EC_{50} estimate for cytosine (21 μM) is similar to that obtained by Stauderman et al., 1998 (26 μM), but not that of Gerzanich et al. (1998) (76 μM). It is noteworthy that the relative efficacy of cytosine is similar in all cases.

The pharmacology of macroscopic currents from cells expressing $\alpha 3\beta 4\alpha 5$ receptors are generally in agreement with previous studies (Gerzanich et al., 1998; Papke et al., 2010). The major discrepancy is in the estimates of the relative efficacy of DMPP. Our experiments (Table 1) demonstrate that the peak current in the presence of DMPP is 61% of that for ACh, whereas Gerzanich et al. (1998) found that the relative efficacy of DMPP was only 13%. In addition, we find that the whole-cell responses from $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ cells show little difference in the rate and extent of desensitization in the presence of ACh. In contrast, Gerzanich et al. (1998) found that the rate of desensitization in the presence of ACh was significantly faster in oocytes expressing $\alpha 3\beta 4\alpha 5$ receptors.

It is not immediately clear what causes the discrepancies, but the difference in expression systems and its effect on

subunit stoichiometry may have influenced the results. In general, our data more closely resemble the results by Stauderman et al. (1998), who like us expressed the receptors in HEK cells, whereas Gerzanich et al. (1998) used *X. laevis* oocytes. It remains to be determined which stoichiometric type is more prevalent in vivo, although for the $\alpha 3\beta 4$ receptor, Lewis et al. (1997) demonstrated that receptors expressed in a mammalian cell line resembled activity from superior cervical ganglion neurons more closely than oocyte-expressed receptors. Other possible reasons for the discrepancies are post-translational modifications such as phosphorylation and glycosylation. These could also depend on the nature of the expression system.

The introduction of the $\alpha 5$ (Asn398) variant had no significant effect on the pharmacological or biophysical properties of the $\alpha 3\beta 4\alpha 5$ receptor. A recent study found that the $\alpha 5$ (Asn398) does not affect sensitivity to ACh, the rate of desensitization or Ca^{2+} permeability of $\alpha 3\beta 4\alpha 5$ receptors (Kuryatov et al., 2011). Our study extends these findings by additionally examining the effect of the $\alpha 5$ (Asn398) variant on channel activation by nicotine, cytisine, DMPP, and epibatidine, by nicotine metabolites NNN, NNK, and cotinine, recovery from desensitization, and single-channel properties (single-channel conductance, intraburst open and closed times). The data indicate that the $\alpha 5$ (Asn398) variant, previously shown to be linked to individual variability in nicotine use, does not modify the major physiological or pharmacological characteristics of the ganglionic $\alpha 3\beta 4\alpha 5$ receptor expressed in HEK cells. However, we note that the variant may act by influencing receptor expression and hence nicotine current amplitude (Frahm et al., 2011). It is also possible that the $\alpha 5$ (Asn398) variant influences nicotine use by acting on the centrally expressed $\alpha 4\beta 2\alpha 5$ receptor. This suggestion is supported by previous work in which it was found that the Ca^{2+} influx was lower for cells expressing $\alpha 4\beta 2\alpha 5$ (Asn398) compared with $\alpha 4\beta 2$ (Asp398) (Bierut et al., 2008). A possible explanation for this lies in the finding that the $\alpha 5$ (Asn398) variant both reduces the Ca^{2+} permeability and increases the desensitization of $\alpha 4\beta 2\alpha 5$ receptors (Kuryatov et al., 2011).

Authorship Contributions

Participated in research design: Steinbach and Akk.
Conducted experiments: Li, McCollum, Bracamontes, and Akk.
Performed data analysis: Li, Steinbach, and Akk.
Wrote or contributed to the writing of the manuscript: Steinbach and Akk.

References

- Akk G (2001) Aromatics at the murine nicotinic receptor agonist binding site: mutational analysis of the α Y93 and α W149 residues. *J Physiol* **535**:729–740.
- Akk G (2002) Contributions of the non- α subunit residues (loop D) to agonist binding and channel gating in the muscle nicotinic acetylcholine receptor. *J Physiol* **544**:695–705.
- Akk G and Steinbach JH (2003) Activation and block of mouse muscle-type nicotinic receptors by tetraethylammonium. *J Physiol* **551**:155–168.
- Bierut LJ (2007) Genetic variation that contributes to nicotine dependence. *Pharmacogenomics* **8**:881–883.
- Bierut LJ, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau OF, Swan GE, Rutter J, Bertelsen S, Fox L, et al. (2007) Novel genes identified in a high-density genome wide association study for nicotine dependence. *Hum Mol Genet* **16**:24–35.
- Bierut LJ, Stitzel JA, Wang JC, Hinrichs AL, Gruzsa RA, Xuei X, Saccone NL, Saccone SF, Bertelsen S, Fox L, et al. (2008) Variants in nicotinic receptors and risk for nicotine dependence. *Am J Psychiatry* **165**:1163–1171.
- Boorman JP, Beato M, Groot-Kormelink PJ, Broadbent SD, and Sivilotti LG. (2003) The effects of $\beta 3$ subunit incorporation on the pharmacology and single channel properties of oocyte-expressed human $\alpha 3\beta 4$ neuronal nicotinic receptors. *J Biol Chem* **278**:44033–44040.
- Brown RW, Collins AC, Lindstrom JM, and Whiteaker P (2007) Nicotinic $\alpha 5$ subunit deletion locally reduces high-affinity agonist activation without altering nicotinic receptor numbers. *J Neurochem* **103**:204–215.
- Chen J and Auerbach A (1998) A distinct contribution of the delta subunit to acetylcholine receptor channel activation revealed by mutations of the M2 segment. *Biophys J* **75**:218–225.
- Chen Q, Fletcher GH, and Steinbach JH (1995) Selection of stably transfected cells expressing a high level of fetal muscle nicotinic receptors. *J Neurosci Res* **40**:606–612.
- Conroy WG and Berg DK (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. *J Biol Chem* **270**:4424–4431.
- Egleton RD, Brown KC, and Dasgupta P (2008) Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. *Trends Pharmacol Sci* **29**:151–158.
- Filatov GN and White MM (1995) The role of conserved leucines in the M2 domain of the acetylcholine receptor in channel gating. *Mol Pharmacol* **48**:379–384.
- Frahm S, Slimak MA, Ferrarese L, Santos-Torres J, Antolin-Fontes B, Auer S, Filkin S, Pons S, Fontaine JF, Tsetlin V, et al. (2011) Aversion to nicotine is regulated by the balanced activity of $\beta 4$ and $\alpha 5$ nicotinic receptor subunits in the medial habenula. *Neuron* **70**:522–535.
- Gerzanich V, Wang F, Kuryatov A, and Lindstrom J (1998) $\alpha 5$ Subunit alters desensitization, pharmacology, Ca^{2+} permeability and Ca^{2+} modulation of human neuronal $\alpha 3$ nicotinic receptors. *J Pharmacol Exp Ther* **286**:311–320.
- Groot-Kormelink PJ, Boorman JP, and Sivilotti LG (2001) Formation of functional $\alpha 3\beta 4\alpha 5$ human neuronal nicotinic receptors in *Xenopus* oocytes: a reporter mutation approach. *Br J Pharmacol* **134**:789–796.
- Gruzsa RA, Johnson EO, Krueger RF, Breslau N, Saccone NL, Chen LS, Derringer J, Agrawal A, Lynskey M, and Bierut LJ (2010) Incorporating age at onset of smoking into genetic models for nicotine dependence: evidence for interaction with multiple genes. *Addict Biol* **15**:346–357.
- Hopp TP, Prickett KS, Price VL, Libby RT, March CJ, Cerretti DP, Urdal DL, and Conlon PJ (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio-Technology* **6**:1204–1210.
- Ifune CK and Steinbach JH (1993) Modulation of acetylcholine-elicited currents in clonal rat pheochromocytoma (PC12) cells by internal polyphosphates. *J Physiol* **463**:431–447.
- Krashia P, Moroni M, Broadbent S, Hofmann G, Kracun S, Beato M, Groot-Kormelink PJ, and Sivilotti LG (2010) Human $\alpha 3\beta 4$ neuronal nicotinic receptors show different stoichiometry if they are expressed in *Xenopus* oocytes or mammalian HEK293 cells. *PLoS One* **5**:e13611.
- Kuryatov A, Berrettini W, and Lindstrom J (2011) Acetylcholine receptor (AChR) $\alpha 5$ subunit variant associated with risk for nicotine dependence and lung cancer reduces ($\alpha 4\beta 2$) $\alpha 5$ AChR function. *Mol Pharmacol* **79**:119–125.
- Labarca C, Nowak MW, Zhang H, Tang L, Deshpande P, and Lester HA (1995) Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. *Nature* **376**:514–516.
- Lewis TM, Harkness PC, Sivilotti LG, Colquhoun D, and Millar NS (1997) The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J Physiol* **505**:299–306.
- Li P, Covey DF, Steinbach JH, and Akk G (2006) Dual potentiating and inhibitory actions of a benz[e]indene neurosteroid analog on recombinant $\alpha 1\beta 2\gamma 2$ GABA_A receptors. *Mol Pharmacol* **69**:2015–2026.
- Mao D, Perry DC, Yasuda RP, Wolfe BB, and Kellar KJ (2008) The $\alpha 4\beta 2\alpha 5$ nicotinic cholinergic receptor in rat brain is resistant to up-regulation by nicotine in vivo. *J Neurochem* **104**:446–456.
- Maus AD, Pereira EF, Karachunski PI, Horton RM, Navaneetham D, Macklin K, Cortes WS, Albuquerque EX, and Conti-Fine BM (1998) Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. *Mol Pharmacol* **54**:779–788.
- McNeill AD (1991) The development of dependence on smoking in children. *Br J Addict* **86**:589–592.
- Nelson ME and Lindstrom J (1999) Single channel properties of human $\alpha 3$ AChRs: impact of $\beta 2$, $\beta 4$ and $\alpha 5$ subunits. *J Physiol* **516**:657–678.
- O'Leary K, Parameswaran N, McIntosh JM, and Quik M (2008) Cotinine selectively activates a subpopulation of $\alpha 3\beta 4\alpha 6$ nicotinic receptors in monkey striatum. *J Pharmacol Exp Ther* **325**:646–654.
- Papke RL, Wecker L, and Stitzel JA (2010) Activation and inhibition of mouse muscle and neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther* **333**:501–518.
- Qin F, Auerbach A, and Sachs F (1996) Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys J* **70**:264–280.
- Ragozzino D, Fucile S, Giovannelli A, Grassi F, Mileo AM, Ballivet M, Alemà S, and Eusebi F (1997) Functional properties of neuronal nicotinic acetylcholine receptor channels expressed in transfected human cells. *Eur J Neurosci* **9**:480–488.
- Saccone NL, Culverhouse RC, Schwantes-An TH, Cannon DS, Chen X, Cichon S, Giegling I, Han S, Han Y, Keskitalo-Vuokko K, et al. (2010) Multiple independent loci at chromosome 15q25.1 affect smoking quantity: a meta-analysis and comparison with lung cancer and COPD. *PLoS Genet* **6**:e1001053.
- Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau O, et al. (2007) Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Hum Mol Genet* **16**:36–49.
- Schuller HM (2007) Nitrosamines as nicotinic receptor ligands. *Life Sci* **80**:2274–2280.
- Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R, and Colquhoun D (1997) Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not

- p>resemble native rat sympathetic ganglion receptors in single-channel behaviour.
- J Physiol*
- 500**
- :123–138.
- Song P and Spindel ER (2008) Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in lung cancer provides a new target for cancer therapy. *J Pharmacol Sci* **106**:180–185.
- Stauderman KA, Mahaffy LS, Akong M, Veliçelebi G, Chavez-Noriega LE, Crona JH, Johnson EC, Elliott KJ, Gillespie A, Reid RT, et al. (1998) Characterization of human recombinant neuronal nicotinic acetylcholine receptor subunit combinations $\alpha 2\beta 4$, $\alpha 3\beta 4$ and $\alpha 4\beta 4$ stably expressed in HEK293 cells. *J Pharmacol Exp Ther* **284**:777–789.
- Ueno S, Zorumski C, Bracamontes J, and Steinbach JH (1996) Endogenous subunits can cause ambiguities in the pharmacology of exogenous gamma-aminobutyric acidA receptors expressed in human embryonic kidney 293 cells. *Mol Pharmacol* **50**:931–938.
- Vernallis AB, Conroy WG, and Berg DK (1993) Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. *Neuron* **10**:451–464.
- Wang JC, Cruchaga C, Saccone NL, Bertelsen S, Liu P, Budde JP, Duan W, Fox L, Grucza RA, Kern J, et al. (2009) Risk for nicotine dependence and lung cancer is conferred by mRNA expression levels and amino acid change in CHRNA5. *Hum Mol Genet* **18**:3125–3135.
- Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, and Kellar KJ (1998) Rat $\alpha 3/\beta 4$ subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. *Mol Pharmacol* **54**:322–333.

Address correspondence to: Gustav Akk, Dept. of Anesthesiology, Campus Box 8054, 660 S. Euclid Ave, St. Louis, MO 63110. Tel: (314) 362-3877; Fax: (314) 362-8571; Email: akk@morpheus.wustl.edu