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Functional characterization of the α5(N398) variant associated with risk for nicotine dependence in the α3β4α5 nicotinic receptor

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

Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO 63110, USA

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Running title: Effect of α5(N398) on α3β4α5 receptor function

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

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Abbreviations: ACh, acetylcholine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; NNN, N-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyrydyl)-1-butanone

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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 asparagine to aspartic acid substitution in the nicotinic receptor α5 subunit at amino acid position 398 (rs16969968; α5(N398)). The α5 subunit is expressed both in the brain and in the periphery. In the brain, it associates with the α4 and β2 subunits to form α4β2α5 receptors. In the periphery, the α5 subunit combines with the α3 and β4 subunits to form the major ganglionic postsynaptic nicotinic receptor subtype. The α3β4α5 receptor regulates a variety of autonomic responses such as control of cardiac rate, blood pressure and perfusion. In this paradigm, the α5(N398) variant may act by regulating autonomic responses which may affect nicotine intake by humans. Here, we have investigated the effect of the α5(N398) variant on the function of the α3β4α5 receptor. The wild-type or variant α5 subunits were coexpressed with the α3 and β4 subunits in HEK 293 cells. The properties of the receptors were studied using whole-cell and single-channel electrophysiology. The data indicate that the introduction of the α5(N398) mutation has little effect on the pharmacology of receptor activation, receptor desensitization, or single-channel properties. We propose that the effect of the α5(N398) variant on nicotine use is not mediated by an action on the physiological or pharmacological properties of the α3β4α5 subtype.

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INTRODUCTION

Smoking leads to over 400,000 premature deaths in the United States annually. Over 45 million people in the U.S. smoke, however, only about one-third of the individuals 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; Grucza et al., 2010; Saccone et al., 2010; Wang et al., 2009). One of these SNPs leads to an asparagine-to-aspartic acid substitution in the nicotinic receptor α5 subunit at the amino acid position 398 (Bierut et al., 2008; Saccone et al., 2007). The α5(D398) is the major allele in all populations studied, and the presence of the N398 variant is associated with a significantly increased risk for increased nicotine use (Bierut et al., 2008; Saccone et al., 2007). It should be noted that the aspartic acid residue at position 398 is highly conserved across species.

The α5 subunit is expressed both in the brain and in the periphery. In the brain it associates with α4 and β2 subunits to form presynaptic α4β2α5 receptors. By some accounts, up to 40 % of epibatidine-labeled nicotinic receptors in the brain contain the α5 subunit (Brown et al., 2007; Mao et al., 2008). The α5 subunit is also highly expressed in the periphery where it combines with the α3 and β4 subunits to form the major postsynaptic nicotinic receptor subtype in the autonomic ganglion cells (Conroy and Berg, 1995; Vernallis et al., 1993). In the α3β4α5 combination, the α5(N398) 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 α3, β4 and α5 subunits are expressed in a number of non-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).

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Here, we have examined the functional effect of the α5(N398) variant on the nicotinic receptor function. The wild-type and variant α5 subunits were coexpressed alongside the peripheral α3 and β4 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 α5(N398) variant has little effect on the pharmacology of the receptor, desensitization, or the major single-channel properties.

METHODS

cDNAs and molecular biology

The experiments were conducted on human embryonic kidney (HEK) 293 cells, transiently or stably expressing combinations of human nicotinic α3, β4, and α5 subunits. We initially created a HEK line stably expressing α3 and β4 subunits. This cell line was used in transient transfections with wild-type and mutant α5 subunits. The cDNAs for the wild-type α3 (accession number NP_000734.2) and β4 (accession number NP_000741.1) subunits were kindly provided by Dr. J. Lindstrom (University of Pennsylvania). A codon optimized human nicotinic α5 subunit cDNA (Accession number BC033639) in pUC57 was obtained from Genscript USA Inc. (Piscataway, NJ). The cDNA was amplified by PCR using primers complementary to the 5' and 3' termini, introducing NotI endonuclease sites at each end of the PCR 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 α5 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 α5FLAG cDNA was mutated with QuikChange to generate the α5(N398), α5(V9'S), and α5(N398+V9'S) mutant clones. The mutated subunits were fully sequenced to confirm that only the desired mutation(s) had been produced.

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Generation of HEK 293 cells stably expressing human nicotinic α3β4 receptors.

HEK 293 cells (CRL-1573: 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 u/ml) and streptomycin (100 µg/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 (1,000,000 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 α3 and β4 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 about 20 hours. 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 µg/ml; GIBCO, Grand Island, NY). 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 (mAb) 35 (Sigma-Aldrich, St. Louis, MO), which binds to an epitope on the extracellular surface of the α3 subunit. We call this procedure "panning". The day before panning, 60 mm Petri dishes were coated with 3 ml of mAb 35 at 25 µg/ml concentration in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 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 ml 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 (HBSS; Mediatech, Manassas, VA) containing 5 % fetal bovine serum. The entire contents of the 15-ml centrifuge tube were dispensed into the pre-coated 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 HBSS

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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 percent confluency. Panning was repeated three times to establish the stable α3β4 cell line used in this study. The cell line was then maintained in HEK growth media with 400 μg/ml G418.

The stable α3β4 cell line was used for transient transfections with wild-type or mutant α5 subunits to express α3β4α5 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 6-18 hours. Electrophysiological experiments commenced the following day.

On the day of experiments we employed a beadbinding technique to select α5 expressing cells for electrophysiology (Ueno et al., 1996). The α5 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, Carlsbad, CA). The cells were incubated with the beads for 5-10 minutes with gentle shaking, and cells expressing the α5 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 (α3β4, 28 cells), -1560 ± 767 pA (α3β4α5, 20), -1981 ± 1031 pA (α3β4α5(N398), 20), -1502 ± 713 pA (α3β4α5(V9'S), 20) and -1431 ± 661 pA (α3β4α5(N398 + V9'S), 19) (mean ± SD). 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 (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES; pH 7.4. The pipet (intracellular) solution contained (in mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES,

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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 ^{o}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 (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES; pH 7.4. The pipet solution contained (in mM): 142 KCl, 5.4 NaCl, 1.7 $MgCl_2$, 1.8 $CaCl_2$ and 10 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, Union City, CA), low-pass filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software (Molecular Devices). Prior to kinetic analysis, the currents were low-pass filtered at 3-5 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;

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www.qub.buffalo.edu). The records were initially analyzed by fitting a simple C ↔ 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, cytisine or 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 ± SD. The fits of averaged concentration-effect curves and exponential curves were conducted using NFIT (The University of Texas, Medical Branch at Galveston), and results are reported as best-fitting value ± estimated 95% interval of the value.

RESULTS

Macroscopic pharmacological profiles of α3β4 and α3β4α5 receptors*

We determined the pharmacological profiles for the α3β4, α3β4α5, and α3β4α5(N398) receptors. The receptors were expressed in HEK 293 cells. The cells were exposed to 1-10,000 μM ACh, 1-300 μM nicotine, 1-300 μM cytisine, or 1-300 μM 1,1-dimethyl-4-phenylpiperazinium (DMPP). In the cases of nicotine, cytisine and DMPP, the responses were normalized to the peak response elicited by 1

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mM ACh from the same cell. Our initial data suggested that there was relatively little difference in responses of receptors containing α3β4 and α3β4α5 subunits, which led to a concern that the α5 subunit was poorly incorporated into functional surface receptors. Accordingly, we inserted a FLAG epitope near the amino terminus of the α5 subunit (see Methods), and selected cells for study which bound small beads coated with antibody to the FLAG epitope. This approach meant that only cells expressing the α5 subunit were studied. To confirm that the α5 subunit was present in the receptors which 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 (Krashia et al., 2010; Labarca et al., 1995).

The data demonstrate that ACh and nicotine have higher efficacy on any of the subunit combinations than cytisine or DMPP, for which the peak responses range from 50 % to 80 % of the ACh response. The EC_{50} for α3β4 receptors activated by ACh was 142 μM, similar to the values observed previously (Gerzanich et al., 1998; Krashia et al., 2010). The addition of the α5 subunit resulted in channels with a slightly reduced EC_{50} (119 μM). A similar leftward shift was previously observed for α3β4α5 receptors expressed in oocytes (Gerzanich et al., 1998). The incorporation of the α5(N398) variant further leftshifted the ACh concentration-response curve (EC_{50} = 57 μM). However, when the midpoints of concentration-response curves from individual cells were compared, the α5(N398) variant was not statistically different from the α3β4α5 (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}s were 16 μM or 19 μM when the V9'S mutation was introduced onto the α5(D398) or α5(N398) 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 α5 subunit in the functional receptors.

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The EC_{50}s for nicotine were tightly grouped (14-23 μM) for the α3β4, α3β4α5 and α3β4α5(N398) receptors. The introduction of the V9'S mutation to the α5 subunit left-shifted the midpoints of the concentration-response curves by 3 to 8-fold. Activation by cytisine resulted in peak currents from α3β4, α3β4α5 and α3β4α5(N398) receptors that were 53 to 65 % of the response to 1 mM ACh. The estimated EC_{50} values were 15-21 μM. The presence of the gain-of-function V9'S mutation enhanced the relative cytisine response to 73-76 %, and shifted the concentration-response curves to lower cytisine 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 8-16 μM for the α3β4, α3β4α5 and α3β4α5(N398) receptors, and 2-5 μM for the receptors containing the α5(V9'S) mutation. The DMPP concentration-response curves suggested that the presence of the α5(N398) variant may enhance the maximal current (Figure 1D, Table 1) compared to α5(D398). To verify this we separately compared responses to 1 mM ACh and 100 μM DMPP from the same set of cells. The relative response to DMPP was 71 ± 5 % (n = 5 cells) and 67 ± 7 % (n = 6 cells) in cells expressing α3β4α5 and α3β4α5(N398) receptors, respectively. We conclude that the α5(N398) variant does not modulate receptor sensitivity to DMPP.

The concentration-response data are summarized in Table 1 and the concentration-response curves for α3β4, α3β4α5, and α3β4α5(N398) receptors are shown in Figure 1. Our overall conclusion is that the presence of the α5(N398) variant has little effect on the agonist concentration-response properties, when compared to the major α5(D398) 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 loss of response, especially at high concentrations. However, there did not appear to be major effects on receptors including either variant of the α5 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 α3β4, α3β4α5 and α3β4α5(N398) receptors, respectively. Accordingly, it

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does not appear that inclusion of the α5(N398) variant had a significant effect on responses to epibatidine.

We also examined receptor activation by cotinine, N-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyrydyl)-1-butanone (NNK). Cotinine is a major metabolite of nicotine, which has a long half-life in the body while 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 two 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 α3β4, α3β4α5 or α3β4α5(N398) receptors. We examined the modulatory effects of the drugs by coapplying NNN or NNK at concentrations up to 100 μM, or cotinine at concentrations up to 1 mM with 30 μM ACh (~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 while 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 α3β4 receptors to 3 ± 4 % of control (n = 4 cells; $p < 0.001$).

Desensitization properties of α3β4, α3β4α5, and α3β4α5(N398) 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

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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 α5 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 phaeochromocytoma 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 α3β4 receptors activated by 1 mM ACh (Table 3). The inclusion of the α5 or α5(N398) 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 α3β4α5 and α3β4α5(N398) 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 presence of the α5 subunit, wild-type or containing the α5(N398) 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 α5 subunit. Cells expressing α3β4 receptors had a 100 to 80 % decay time of 0.9 s. When the α5 subunit was included in receptor complexes, the decay time increased to 8.6 s. The introduction of the α5(N398) variant did not further influence receptor desensitization (τ = 9.6 s). Sample current traces are shown in Figure 2 and the summary of the analysis including statistical significances are given in Table 3.

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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 (Figure 3). For the majority of receptor-agonist combinations, best fits for the time courses contained two exponential components. For the α3β4 receptor the two components had mean durations of approximately 5 s 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 α5 subunit and the introduction of the α5(N398) variant had a relatively small effect on the recovery time course for desensitization induced by ACh (Table 4). Interestingly, the recovery time course for α3β4α5(N398) 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 α3β4 and α3β4α5 receptors, and similar to the weighted time constant from these receptors.

To gain more insight into the effect of the α5 subunit on recovery, we compared fractional recovery following a 30 s washout. Following desensitization by 1 mM ACh, the mean fractional recovery was 79 ± 15 % (n = 6 cells), 79 ± 11 % (n = 5) and 69 ± 13 % (n = 5) for cells expressing α3β4, α3β4α5 and α3β4α5(N398) receptors. The effect of the α5(N398) variant was statistically insignificant. Following 100 μM nicotine, the α3β4 receptors recovered to 65 ± 6 % (n = 6 cells) of control following a 30 s washout. Recovery was 80 ± 18 % (n = 3) for α3β4α5 receptors and 58 ± 13 % (n = 4) for α3β4α5(N398) receptors. The differences were not statistically significant indicating that the presence of a single component in the recovery time course from α3β4α5(N398) receptors does not result in significant changes in recovery from desensitization.

Single-channel currents from α3β4 and α3β4α5* receptors

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We investigated the effect of subunit composition and the α5(V9'S) mutation on single-channel parameters. The α3β4 stable cell line was supertransfected with the α5, α5(N398), α5(V9'S), or α5(N398+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. Due to its relative scarcity the higher conductance class was not characterized.

Sample recordings are shown in Figure 4. The intraburst open time histograms were adequately fitted to a single exponential. The α3β4 receptors had a mean open duration of 10.7 ± 4.0 ms (n = 4 patches). In α3β4α5 receptors, the mean open duration was 3.5 ± 1.0 ms (n = 3 patches). As expected the introduction of the α5(V9'S) mutation prolonged channel openings, increasing the mean open duration to 9.7 ± 1.8 ms (n = 3 patches). The presence of the α5(N398) variant had no significant effect on the mean open time (4.7 ± 0.9 ms; n = 3 patches) compared to α3β4α5. In the receptor containing the α5(N398+V9'S) double mutation, the mean open time was 9.7 ± 1.4 ms (n = 4 patches). We conclude that the α5(N398) variant is without effect on the open time durations, regardless of the presence of the background α5(V9'S) mutation. Conversely, the data also show that the V9'S mutation similarly affects the open durations from receptors containing both variants of the α5 subunit. Cumulative plots of the open durations are shown in Figure 4F. The reduction in open time following introduction of α5 or α5(N398) is highly significant ($p < 0.001$ for comparison to α3β4; Kolmogorov-Smirnov 2-tailed test). The differences between α5 and α5(V9'S), and between α5(N398) and α3β4α5(N398+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 1-3 ms. The presence of the α5 subunit did not affect the closed time distributions. The closed time data are summarized in Table 5.

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We determined the slope conductance of single-channel activity by measuring the current-voltage relationship at membrane potentials of -100 mV to -25 mV. The single-channel conductances were indistinguishable: α3β4 receptors 34.0 ± 2.1 pS (n = 4 patches), α4β4α5 33.7 ± 3.6 pS (n = 3 cells); α3β4α5(N398) 32.8 ± 1.9 pS (n = 4 cells).

A previous study employing outside-out patches from *Xenopus* oocytes expressing α3β4 or α3β4α5 receptors found a single-channel conductance of 31 pS and 36 pS, respectively (Nelson and Lindstrom, 1999). Nicotinic currents from rat superior cervical ganglion cells, where α3β4 receptors form a major nicotinic receptor component, show a single-channel conductance of 35-37 pS (Sivilotti et al., 1997). Rat α3β4 receptors expressed in BOSC 23 cells predominantly show a 34 pS conductance class (Ragozzino et al., 1997). Overall, our findings are similar to the previously published single-channel data on wild-type α3β4 and α3β4α5 receptors (e.g.,(Boorman et al., 2003; Lewis et al., 1997; Nelson and Lindstrom, 1999). Our single-channel data indicate that the α5(D398) and α5(N398) variants behave similarly in single-channel recordings.

DISCUSSION

The neuronal nicotinic α5 subunit does not express as a homooligomer nor can it substitute for other neuronal α subunits which combine with a β subunit to form functional channels. Rather the α5 subunit acts as an accessory subunit combining with, and in some cases modulating, other αβ heterooligomeric receptors. The α5 subunit is expressed in the brain where it contributes to the α4β2α5 receptor, and in the periphery where it combines with α3 and β4 subunits to form the major ganglionic postsynaptic receptor (Conroy and Berg, 1995; Vernallis et al., 1993). In addition, the nicotinic α5 subunit is expressed in lung epithelial cells with α3 and β4, and in small cell and non-small cell lung cancer cell lines (Egleton et al., 2008; Maus et al., 1998; Song and Spindel, 2008). We examined the effects of including two sequence variants of the α5 subunit on the physiological and

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pharmacological properties of α3β4 receptors. The minor allele (α5(N398)) is associated with increased risk of developing nicotine dependence (Bierut et al., 2008; Saccone et al., 2007). 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 α3β4, α3β4α5, and α3β4α5(N398) receptors. We also generated and studied receptors containing a valine-to serine mutation in the second membrane-spanning domain (V9'S) of the α5 subunit. This gain-of-function mutation acted as a functional reporter for the expression and incorporation of the α5 subunit.

Our findings indicate that the α5(N398) variant has no functional effect in the α3β4α5 receptor, compared to the major allele, insofar as covered by the tests that we employed. Specifically, the variant was without effect on channel activation by ACh, nicotine, cytisine 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 intracluster open and closed times and single-channel conductance.

Given its passive nature, the expression and incorporation of the α5 subunit cannot be determined simply from the observation of a functional response. Nor are we aware of any specific antibodies to the α5 subunit or a pharmacological approach to eliminate non-α5 containing receptors. Accordingly, we engineered a FLAG epitope tag at the aminoterminus of the subunit. Cells expressing the α5 subunit on the surface were selected by beadbinding. Although this approach does not guarantee the presence of the α5 subunit in each receptor-complex, bead binding indicates the incorporation of the α5 subunit in, at least, some surface receptors. The presence of the α5 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 α5 subunit contained the V9'S mutation in the second membrane-spanning domain. Original studies on the

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related muscle-type nicotinic receptor showed 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 (Chen and Auerbach, 1998; Filatov and White, 1995; Labarca et al., 1995). This results in a leftward shift in the agonist concentration-response curve. Accordingly, we used this approach to probe for the presence of the α5 subunit. Our data indicate that the introduction of the V9'S mutation in the α5 subunit prolongs the mean open duration in single-channel recordings (Figure 4) and shifts the macroscopic concentration-response curves to lower agonist concentrations (Table 1). Importantly, the presence of the N398 variant on the α5(V9'S) background did not influence macroscopic parameters or the properties of single-channel currents, compared to the major allele. These findings are important because in these experiments the presence of the α5 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 α3β4α5 receptors expressed in *Xenopus* 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 α3β4 receptors exist in two stoichiometries: one with 2 α3 subunits and 3 β4 subunits, the other containing 3 α3s and 2 β4s (Krashia et al., 2010). The two stoichiometric isoforms differed in their relative sensitivity to DMPP (the 3 α isoform was more sensitive) and single-channel properties (the 3 α isoform had a larger single-channel conductance). Examination of our data suggests that the stable α3β4 cell line utilized in this study expresses receptors in the 3:2 (α3:β4) stoichiometry. The ratio of EC_{50}-s for ACh vs. DMPP is 7.7 (Table 1), similar to the 10-fold difference found by Krashia et al. (2010) for the 3 α isoform. However, our single-channel conductance estimate for α3β4 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

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results from differences in recording conditions (cell-attached vs. outside-out) or indicates that our cells express both isoforms.

Our estimates for activation properties of the α3β4 receptor show similarites as well as differences with previous studies. For example, our estimate for the EC_{50} for DMPP (16 μM) is close to the values previously obtained (10-18 μM; (Gerzanich et al., 1998; Stauderman et al., 1998)). Similarly, our estimate for the EC_{50} for ACh (142 μM) agrees with Gerzanich et al. (163 μM) and Papke et al. (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 $^{86}Rb^{+}$ flux yielded EC_{50}s of 28 and 114 μM for nicotine and ACh, respectively (Xiao et al., 1998). Our EC_{50} estimate for cytisine (21 μM) is similar to that obtained by Stauderman et al. (26 μM), but not Gerzanich et al (76 μM). Interestingly, the relative efficacy of cytisine is similar in all cases.

The pharmacology of macroscopic currents from cells expressing α3β4α5 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 α3β4 and α3β4α5 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 α3β4α5 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 *Xenopus* oocytes. It remains to be determined which stoichiometric type is more prevalent in vivo, although for the α3β4 receptor, Lewis et al. (1997)

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demonstrated that receptors expressed in a mammalian cell line more closely, than oocyte-expressed receptors, resembled activity from superior cervical ganglion neurons. Other possible reasons for the discrepancies are posttranslational modifications such as phosphorylation and glycosylation. These could also depend on the nature of the expression system.

The introduction of the α5(N398) variant had no significant effect on the pharmacological or biophysical properties of the α3β4α5 receptor. A recent study found that the α5(N398) does not affect sensitivity to ACh, the rate of desensitization or Ca^{++} permeability of α3β4α5 receptors (Kuryatov et al., 2011). Our study extends these findings by additionally examining the effect of the α5(N398) variant on channel activation by nicotine, cytisine, DMPP and epibatidine, 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 α5(N398) variant, previously shown to be linked to individual variability in nicotine use, does not modify the major physiological or pharmacological characteristics of the ganglionic α3β4α5 receptor expressed in HEK cells. However, we note that the variant may act by influencing receptor expression and, hence, nicotinic current amplitude (Frahm et al., 2011). It is also possible that the α5(N398) variant influences nicotine use by acting on the centrally expressed α4β2α5 receptor. This suggestion is supported by previous work in which it was found that the Ca^{2+} influx was lower for cells expressing α4β2α5(N398) compared to α4β2(D398) (Bierut et al., 2008). A possible explanation for this lies in the finding that the α5(N398) variant both reduces the Ca^{2+} permeability and increases the desensitization of α4β2α5 receptors (Kuryatov et al., 2011).

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Steinbach and Akk.

Conducted experiments: Li, McCollum, Bracamontes, and Akk.

Contributed new reagents or analytic tools: N/A

Performed data analysis: Li, Steinbach, and Akk

Wrote or contributed to the writing of the manuscript: Steinbach and Akk.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Summary of activation concentration-response curves. The plots show concentration-response curves for ACh (**A**), nicotine (**B**), cytisine (**C**), and DMPP (**D**) for HEK cells stably expressing human α3β4 receptors, or stable α3β4 cells transiently transfected with wild-type or variant (N398) α5 subunits. The α5 subunits contained a FLAG epitope inserted to the aminoterminus of the subunit. Cells expressing the α5 subunit were selected with immunobeads coated with anti-FLAG antibody. Each data point represents mean ± SEM from 4 to 7 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.

Figure 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 α5 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.

Figure 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 following washout in bath solution for 1-120 s. (**A**) Sample recordings from cells expressing α3β4α5 (top) or α3β4α5(N398) (bottom) receptors. The two traces show recovery following washout for 2 s (left) or 30 s (right). Recovery was calculated as the ratio of second peak minus residual current (grey 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 Figure 2. This is so because the rate and extent of desensitization correlated with recording time. (**B**) Recovery time courses were measured for α3β4, α3β4α5, and α3β4α5(N398) receptors. The curves were fitted to a single exponential (α3β4α5(N398) + nicotine) or the sums of two exponentials (everything else). The fitting results are given in Table 4.

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Figure 4. Single-channel currents from the wild-type and mutant receptors activated by 3 μM ACh. (A) The currents were recorded from a HEK cell stably expressing the human α3β4 receptors. The stably expressing α3β4 cells were used in transient transfections with wild-type (**B**) and mutant (**C-E**) α5 subunits. The wild-type and mutant α5 subunits contained a FLAG epitope inserted at the amino-terminus of the subunit. Cells expressing the α5 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 α5 subunit results in briefer open time durations (left panel). The introduction of the V9'S mutation to the α5 subunit results in longer open time durations (right panel).

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Agonist	Parameter	α3β4	α3β4α5	α3β4α5(N398)	α3β4α5(V9'S)	α3β4α5(N398+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 cells	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 cells	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 cells	5	5	5	5	4

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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 cells	5	5	3	5	4

Table 1. Activation properties of the human α3β4, α3β4α5, and α3β4α5* receptors. The table gives the concentration-response data for the α3β4, α3β4α5, α3β4α5(N398) α3β4α5(V9'S) and α3β4α5(N398+V9'S) receptors. The parameters are estimated by fitting data from n cells with the Hill equation, and the mean ± SD 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} the concentration producing a half-maximal response and n_H the Hill coefficient for the fit. Figure 1 shows the averaged concentration response relationships for the cells.

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Receptor	NNN	NNK	cotinine
α3β4	92 ± 10 % ^{ns}	92 ± 12 % ^{ns}	53 ± 9 % ***
α3β4α5	96 ± 11 % ^{ns}	96 ± 4 % ^{ns}	60 ± 10 % ***
α3β4α5(N398)	88 ± 5 % **	95 ± 2 % **	52 ± 12 % ***

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 ± SD of control currents from 5 to 8 cells for each condition. The statistical significance applies to comparison with control currents. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

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Receptor	Agonist	100 to 50 % decay time	Residual current
α3β4	ACh	15 ± 12 s	29 ± 23 %
α3β4α5	ACh	13 ± 6 s ^{ns}	25 ± 14 % ^{ns}
α3β4α5(N398)	ACh	17 ± 3 s ^{ns,ns}	29 ± 7 % ^{ns,ns}
Receptor	**Agonist**	**100 to 80 % decay time**	**Residual current**
α3β4	nicotine	0.9 ± 0.6 s	9 ± 3 %
α3β4α5	nicotine	8.6 ± 6.2 s *	50 ± 25 % **
α3β4α5(N398)	nicotine	9.6 ± 3.7 s ***,^{ns}	48 ± 15 % ***,^{ns}

Table 3. The effect of the α5 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 ± SD from 5-8 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 α3β4 and α3β4α5 receptors. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

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Receptor	Agonist	τ1 (fraction)	τ2
α3β4	ACh	4.8 ± 0.8 s (55 %)	89 ± 13 s
α3β4α5	ACh	2.2 ± 1.8 s (66 %)	49 ± 32 s
α3β4α5(N398)	ACh	3.5 ± 2.0 s (45 %)	59 ± 16 s
Receptor	**Agonist**	**τ1 (fraction)**	**τ2**
α3β4	nicotine	3.9 ± 1.4 s (40 %)	55 ± 8 s
α3β4α5	nicotine	8.8 ± 1.9 s (73 %)	66 ± 31 s
α3β4α5(N398)	nicotine	36 ± 3 s (100 %)	N/A

Table 4. The effect of the α5 subunit on recovery from desensitization. The receptors were exposed to 30 s pulses of 1 mM ACh or 100 μM nicotine, eliciting channel desensitization. Recovery from desensitization was tested with brief (3 s) pulses at 1-120 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 4-8 cells.

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Receptor	CT1 (ms)	Fraction CT1	CT2 (ms)	Fraction CT2
α3β4	0.11 ± 0.05	0.88 ± 0.05	1.6 ± 0.5	0.12 ± 0.05
α3β4α5	0.11 ± 0.03	0.91 ± 0.06	0.8 ± 0.3	0.09 ± 0.06
α3β4α5(N398)	0.12 ± 0.04	0.80 ± 0.18	0.8 ± 0.4	0.20 ± 0.18
α3β4α5(V9'S)	0.10 ± 0.02	0.88 ± 0.07	2.6 ± 1.7	0.12 ± 0.07
α3β4α5(N398+V9'S)	0.10 ± 0.01	0.96 ± 0.001	1.2 ± 0.6	0.04 ± 0.001

Table 5. The effect of the α5* subunit on the intraburst closed time properties. The intraburst activity was fitted to the sums of two exponentials. The data are mean ± SD from 3-4 patches. The columns give receptor subunit composition, and the mean durations and fractions of the two closed time components.

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Figure 1

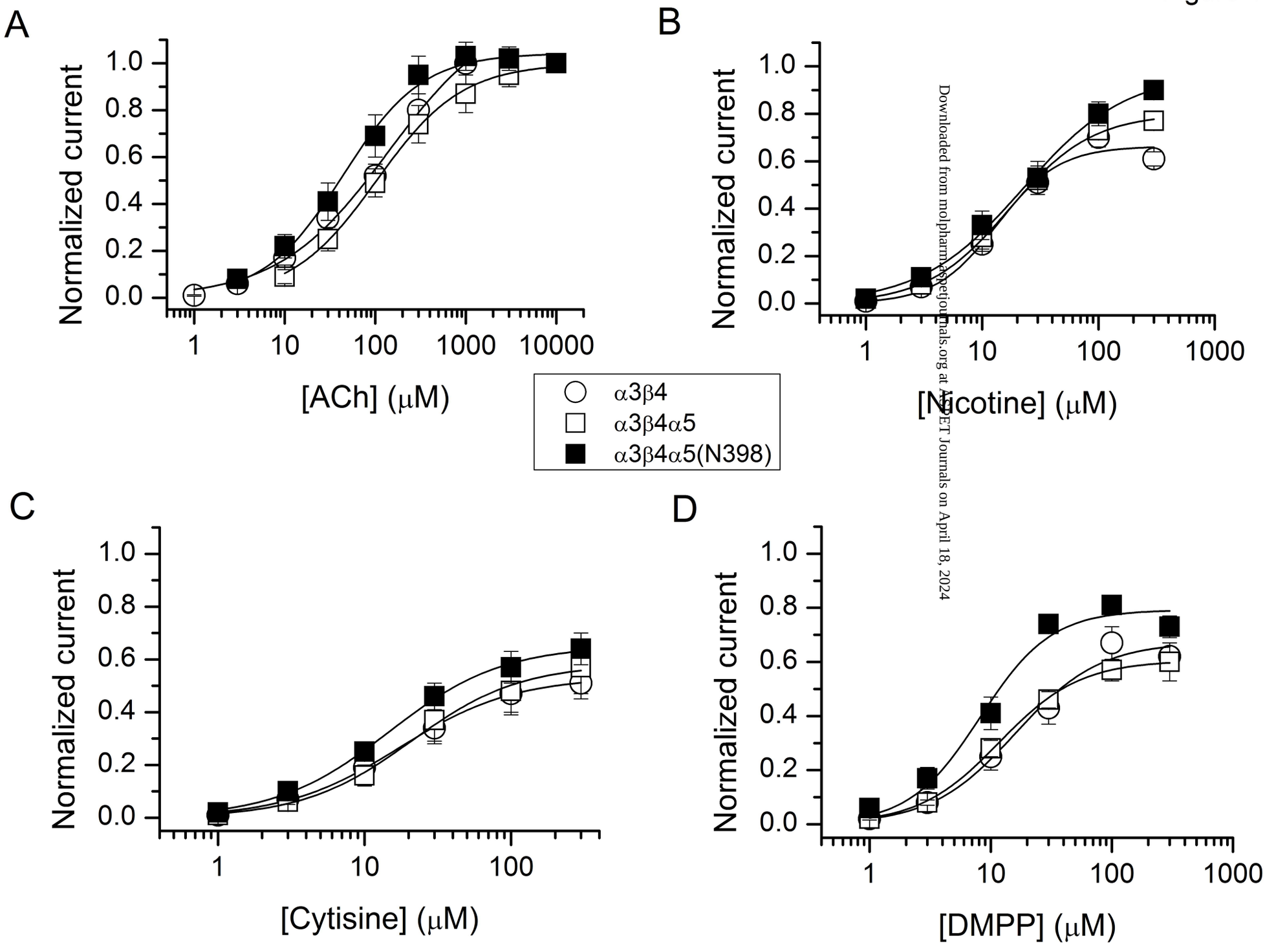

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Figure 2

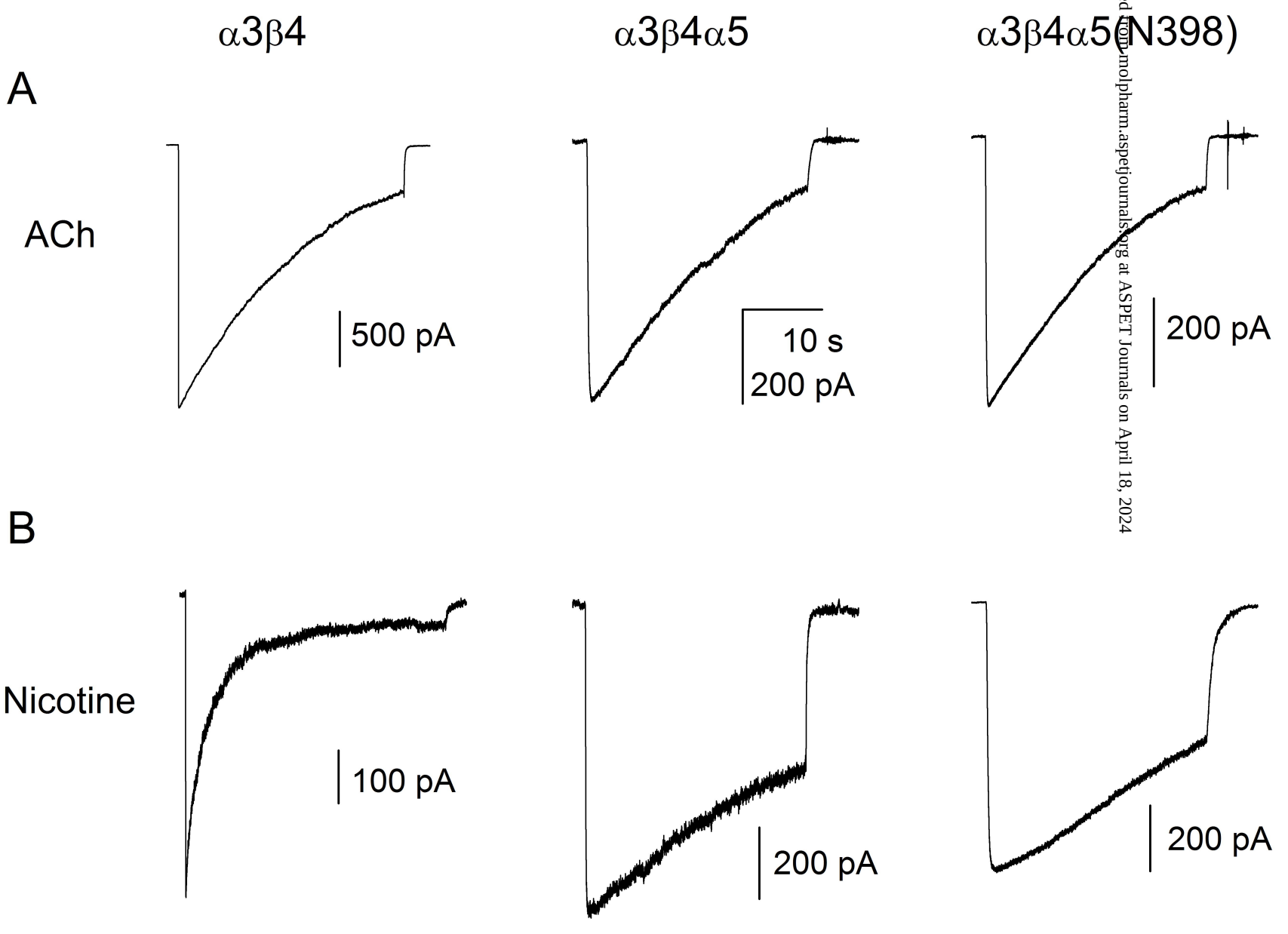

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Figure 3

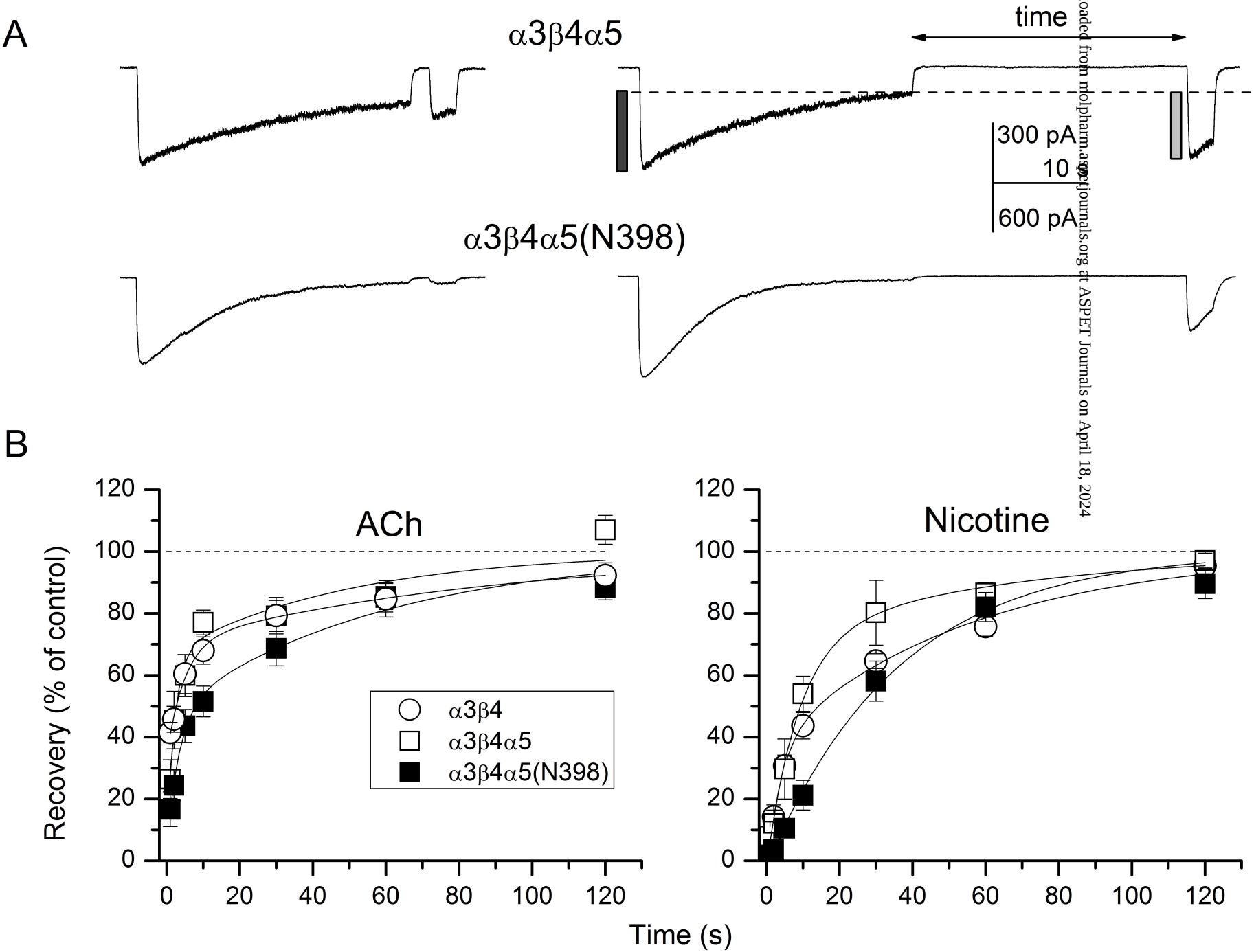

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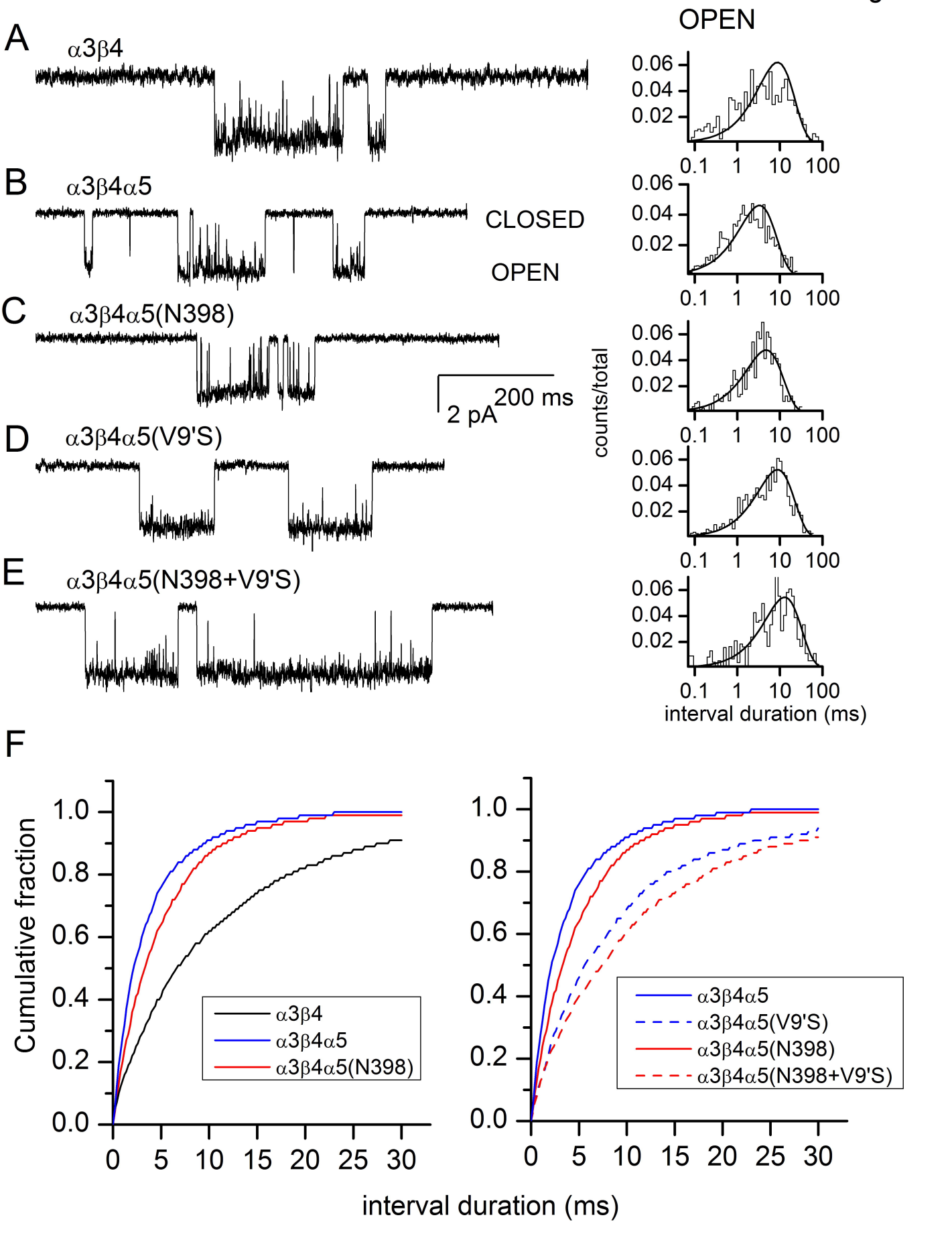
Figure 4


A
α3β4
B
α3β4α5
CLOSED
OPEN
C
α3β4α5(N398)
2 pA
200 ms
D
α3β4α5(V9'S)
E
α3β4α5(N398+V9'S)
OPEN
counts/total
0.06
0.04
0.02
0.1
1
10
100
interval duration (ms)
F
Cumulative fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
α3β4
α3β4α5
α3β4α5(N398)
α3β4α5(V9'S)
α3β4α5(N398+V9'S)
interval duration (ms)