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Untranslated Region Dependent Exclusive Expression of High-Sensitivity Subforms of α4β2 and α3β2 Nicotinic Acetylcholine Receptors

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Running title: High-sensitivity subforms of α4β2 and α3β2 nAChR

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Abbreviations: A-163554 ((R)-2-Chloro-3-(5,5-dimethyl-hexa-1,3-dienyl)-5-(pyrrolidin-2ylmethoxy)pyridine dihydrochloride); A-162035 ((R)-2-chloro-3-phenyl-5-(pyrrolidin-2-ylmethoxy)-pyridine hydrochloride); A-168939 ((R)-5-chloro-6-(2-pyridin-4-yl-vinyl)-2-pyrrolidin-2-yl-furo[3,2-b]pyridine dihydrochloride); acetylcholine (ACh); chlorisondamine (CI); dihydro-β-erythroidine (DHβE); mecamylamine (Mec); methyllycaconitine (MLA); nicotinic acetylcholine receptor (nAChR); open reading frame (ORF); *d*-tubocurarine (dTC); untranslated region (UTR);

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Abstract

α4β2 nicotinic acetylcholine receptors are recognized as the principal nicotine binding site in brain. Recombinant α4β2 nAChR demonstrate biphasic concentration-response relationships with low- and high-EC_{50} components. This study shows that untranslated regions (UTR) can influence expression of high-sensitivity subforms of α4β2 and α3β2 nAChR. Oocytes injected with α4 and β2 RNA lacking UTR expressed biphasic concentration-response relationships for acetylcholine with high-sensitivity EC_{50} values of 0.5 to 2.5 μM (14-24% of the population) and low-sensitivity EC_{50} values of 110-180 μM (76-86%). In contrast, message with UTR expressed exclusively the high-sensitivity α4β2 nAChR subform with an acetylcholine EC_{50} value of 2.2 μM. Additional studies revealed pharmacologic differences between high- and low-sensitivity α4β2 subforms. While the antagonists dihydro-β-erythroidine (IC_{50} 3-6 nM) and methyllycaconitine (IC_{50} 40-135 nM) were not selective between high-and low-sensitivity α4β2, chlorisondamine, mecamylamine and *d*-tubocurarine were, respectively, 100-, 8-, and 5-fold selective for the α4β2 subform with low-sensitivity to acetylcholine. Conversely, agonists that selectively activated the high-sensitivity α4β2 subform with respect to efficacy as well as potency were identified. Further, two of these agonists were shown to activate mouse brain α4β2 as well as the ferret high-sensitivity α4β2 expressed in *Xenopus* oocytes. Using UTR-containing RNA, exclusive expression of a novel high-sensitivity α3β2 nAChR was also achieved. These studies (a) provide further evidence for the existence of multiple subforms of α4β2 nAChR, (b) extend that to α3β2 nAChR, (c) demonstrate UTR influence on β2-containing

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nAChR properties, and (d) reveal compounds that interact with $\alpha4\beta2$ in a subform-selective manner.

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Introduction

Nicotinic acetylcholine receptors (nAChRs) are a diverse group of ligand-gated ion channels found in brain and spinal cord, autonomic, enteric and sensory nervous systems, skeletal muscle, cochlea and a several non-neuronal cell types (Champtiaux and Changeux, 2004; Alkondon and Albuquerque, 2004; Gotti and Clementi, 2004; Hogg and Bertrand, 2004). These receptors are defining members of the pentameric superfamily including $5HT_3$, $GABA_A$ and glycine receptors. Functional receptors are comprised by at least one "α" subunit which contains signature sequences required for binding and channel activation. However, most nAChR also require non-α subunits in order to form a functional complex, which together with the pentameric structure could permit formation of multiple functionally distinct nAChR from even just two different subunits, e.g. $\alpha4_{(2)}\beta2_{(3)}$ and $\alpha4_{(3)}\beta2_{(2)}$ (Zhou et al., 2003). In mammalian brain, nine subunits predominate - α2 through α7 and β2 through β4, and among these only α7 can form homomeric functional pentamers (Champtiaux and Changeux, 2004; Gotti and Clementi, 2004).

Despite the potential huge diversity of nAChR, most CNS functions have been ascribed to α4β2, α3-containing (α3*), α6*, and α7 nAChR. In particular, about 90% of the high-affinity nicotine binding sites in rat brain comprise α4β2 (Champtiaux et al., 2003; Zoli et al., 1995; Flores et al., 1992; Whiting et al., 1987; Clarke et al., 1985). Functionally, native α4β2 nAChR EC_{50} values for nicotine and the neurotransmitter acetylcholine are in the low-micromolar range (Marks et al., 1999; Alkondon and Albuquerque, 1993; Marks et al., 1993), 1-2 orders of magnitude lower than for other nAChR and consistent with higher-affinity binding to α4β2. In contrast, recombinant α4β2 expressed in oocytes and mammalian cell lines have demonstrated variable

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functional potencies for acetylcholine and nicotine with lower sensitivity EC_{50} values > 40 μM (Papke et al., 2000; Sabey et al., 1999; Chavez-Noriega et al., 1997; Gopalakrishnan et al., 1996) as well as the higher sensitivity ≤ 3 μM EC_{50} values (Labarca et al., 2001; Olale et al., 1997; Kuryatov et al., 1997; Gopalakrishnan et al., 1996; Buisson et al., 1996; Papke and Heinemann, 1994; Court et al., 1994). Indeed, individual cells may express both high- and low-sensitivity forms of recombinant α4β2 and α4β4 (Houlihan et al., 2001; Covernton and Connolly, 2000) in a proportion that may be influenced by α4 polymorphism (Kim et al., 2003), by β2 content (Nelson et al., 2003; Zhou et al., 2003; Buisson and Bertrand, 2001; Zwart and Vijverberg, 1998), or by prolonged (overnight) exposure to nicotine or low temperature (Nelson et al., 2003; Buisson and Bertrand, 2001). However, it is not clear whether low- as well as high-sensitivity α4β2 nAChR are expressed in CNS, what their respective roles in behavior or development may be, nor how the proportion of high- and low-sensitivity forms may be regulated apart from chronic exposure to nicotine.

In this study, we present evidence that untranslated regions (UTR) of the nAChR transcripts influence the expression of high- and low-sensitivity nAChR to the extent of permitting exclusive expression of the high-sensitivity α4β2 nAChR subform. This property does not appear to be limited to α4β2 nAChR, but extends at least to α3β2 nAChR as well.

Among the various nAChR, α4β2 are unusual in that they are potentiated rather than inhibited by the neuroactive steroid 17β-estradiol (Curtis et al., 2002; Nakazawa and Ohno, 2001) through a mechanism involving the carboxy-terminus of the α4 subunit (Paradiso et al., 2001). Estradiol also potentiated ferret α4β2 nAChR, and with apparently greater effect on the high-sensitivity

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subform. Thus, α4β2 physiology may be regulated through selective modulation by endogenous substances as well as through expression of nAChR with differing sensitivity to the neurotransmitter acetylcholine.

We also evaluated the selectivity of several antagonists and agonists to identify compounds that may be useful for examining the roles of high- and low-sensitivity α4β2 nAChR. With both high-sensitivity and mixed-sensitivity forms of α4β2, dihydro-β-erythroidine (DHβE) and methyllycaconitine were potent antagonists but did not appear to distinguish between the high- and low-sensitivity subforms. In contrast, mecamylamine, *d*-tubocurarine and chlorisondamine were 8-, 5- and 100-fold selective for the low-sensitivity form. None of the antagonists examined was selective for the high-sensitivity α4β2, which, in contrast, is the form more sensitive to acetylcholine. On the other hand, some agonists did appear to be very selective for the high-sensitivity α4β2 nAChR subform. Two of these agonists were shown to be active at mouse brain α4β2 nAChR as well as at ferret high-sensitivity α4β2 nAChR expressed in oocytes, supporting the idea that the high-sensitivity α4β2 nAChR subform is expressed in brain.

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Materials and Methods

Total RNA was prepared from ferret brain (ABS, Wilmington, DE) using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Poly-A+ RNA was isolated using the Oligotex mRNA system (Qiagen, Valencia, CA). Two different methods were employed in cloning the nAChR subunits. One method involved identification of a full-length clone from a cDNA library, while the other utilized standard PCR techniques to amplify fragments. The cDNA library screening utilized for α4 and β2 provided coding sequence and genomic 5'- and 3'-untranslated regions (UTR) while the PCR methodology utilized for α3, α4 and β2 generated coding sequence without the UTR.

Isolation of α4 and β2 from a cDNA library

cDNA was synthesized from ferret brain poly-A+ RNA using the Orient Express kit with random primers, ligated to EcoRI / HindIII linkers (Novagen, Madison, WI) and digested with EcoRI + HindIII (New England Biolabs, Beverly, MA). The cDNA was fractionated on a sucrose gradient to remove material smaller than 500 bp. The vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA) was digested with EcoRI and HindIII, treated with calf intestinal alkaline phosphatase and purified over a Chromaspin-TE 1000 column (Clontech, Palo Alto, CA). Vector and cDNA were ligated with a Novagen DNA Ligation Kit and transformed into ElectroMax DH10B cells (Invitrogen) by electroporation. The electroporation mixture was diluted to approximately 1000 transformants / ml in autoclaved 2% tryptone/1% yeast extract/1% NaCl/0.3% SeaPrep agarose (BioWhittaker, Rockland, ME), equilibrated to 37°C and supplemented with 100 mg/ml

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ampicillin. Aliquots (40 ml) were poured into sterile 50 ml tubes, chilled in iced water for 30 minutes to solidify the agarose and incubated at 30°C for 2 days. Tubes were inverted several times to mix colonies, and a small aliquot from each tube was stored at –80°C in 15% glycerol. The remaining cells were centrifuged, and plasmid DNA isolated with REAL prep 96 kit (Qiagen, Valencia, CA). A total of 384 library aliquots (4, 96-well plates) were prepared, representing approximately 20 million clones.

For library screening, plasmid DNA was denatured with base and spotted on positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN) with a 96-pin device (V&P Scientific, San Diego, CA). The membranes were neutralized and the DNA fixed by UV exposure (Stratalinker; Statagene, La Jolla, CA). Membrane replicates were then hybridized individually to various oligonucleotides that had been labeled with T4 polynucleotide kinase (Invitrogen) and γ-^{32}P-ATP, washed at varying stringencies and exposed at –80°C with Kodak Biomax intensifying screens and Biomax film. The following oligonucleotide probes were prepared, following a design based upon homology to published nAChR subunits and to partial ferret sequence data derived from short PCR fragments:

Oligonucleotide 1: GCCGCTCTTCTACACCATCAACCTCATC
(highly conserved for all α and β nAChR subunits)

Oligonucleotide 2: GAACGGTTGCTGAAGACACTCTTCTCCGGCTACAACAAGTGGTC
(ferret α4, N terminal half)

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Oligonucleotide 3: GGCGGCTCATCGAGTCCATGCACAAGGTGGCCAGCGCCCC

(ferret α4, C terminal half)

Oligonucleotide 4: GAGCGGCTAGTGGAGCATCTCCTGGACCCCTCCCGGTACAACAAG

(ferret β2, N terminal half)

Oligonucleotide 5: ACCATCGGCATGTTCCTGCAGCCTCTCTTCCAGAACTACAC

(human β2, C terminal half).

Based upon hybridization signals, individual library aliquots believed to contain full-length α4 and β2 subunit cDNA clones were identified. Colonies from each were plated onto agar, grown, transferred to nylon membranes and screened with oligonucleotide probes. For α4 a mixture of oligonucleotides 1, 2 and 3 was used; for β2 a mixture of oligonucleotides 1, 4 and 5 was used. Individual colonies were identified and characterized. All α4 colonies were found to contain identical inserts for the complete coding sequences plus 5' and 3' non-coding regions. There were two different cDNA inserts for β2; one began with 5' non-coding sequences and extended towards the middle, while the other began in the middle coding region and ended in 3' non-coding sequences. Since there were several hundred nucleotides of overlap between the latter two clones that included a unique BsgI restriction site, a series of restriction digestions and ligations were used to produce a full-length β2 clone.

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Isolation of α3, α4 and β2 cDNAs by PCR

cDNA prepared from either total RNA or poly-A+ RNA was amplified by PCR using the Superscript II Preamplification System (Invitrogen) and either oligo(dT) or random hexamer primers. First strand cDNA synthesis was performed according to the manufacturer's instructions. Briefly, the RNA was primed with either random hexamers or oligo (dT) in the presence of dNTPs and reactions initiated by the addition of 50U Superscript II RT. After termination of the reaction the remaining RNA template was removed by treatment with 2U of RNase H, and partial cDNAs were then amplified by PCR using gene specific primers. Primers were designed to correspond to areas that are divergent from sequences of other nAChR subunits but show relatively good homology between human and rat cDNA sequences of the desired nAChR subunit. In some instances degenerate primers were used. DNA sequences were amplified by PCR using either Advantage HF, Advantage HF2 (BD Biosciences Clontech, Palo Alto, CA), or Amplitaq Gold (Perkin Elmer, Boston, MA) polymerases. Briefly, for β2, after initial template denaturation for 3 minutes at 94°C, amplification was performed with thermal cycles of 94°C for 30 seconds, followed by 68°C for 3 minutes for 35 cycles (2-step PCR), followed by a final extension at 68°C for 7 minutes. For α4 and α3, the template was denatured for 30 seconds at 94°C and amplification was performed with thermal cycles of 94°C for 15 seconds, followed by 68°C for 3 minutes for 35 cycles, followed by a final extension at 68°C for 7 minutes. In some instances, PCR was performed in the presence of 5% dimethylsulfoxide (DMSO) or by using Advantage-GC2 (BD Biosciences Clontech), when specific GC-rich areas of the cDNAs were unobtainable under more standard PCR conditions. A 20 μl aliquot of the reaction was run on a 1% agarose gel and PCR products of the expected size were extracted

using the QIAquick kit (Qiagen), cloned into the pCR 2.1-TOPO vector (Invitrogen), and expanded using One Shot TOP 10 chemically competent E. coli (Invitrogen) in preparation for sequencing. DNA sequences were identified and confirmed with overlapping sequences generated from different PCR primer sets. For the α3 nAChR subunit, four overlapping partial cDNA clones were used to construct a full-length clone, for the α4 subunit three overlapping clones were used, and for the β2 cDNA four overlapping clones were used. Primer sequences used to generate these partial cDNAs were as follows.

α3 nAChR primers

Set 1: 5'-CTCCAGGTCTGGGGTCTGCGCTG-3' (sense),
5'-GCTTTGGTCTTGTCGTCCACCTGG-3' (antisense)

Set 2: 5'-GCCAGTGGCCAGGGCCTCAGAGGC-3' (sense),
5'-CCCAGTAGTCCTTGAGGTTCATGG-3' (antisense)

Set 3: 5'-CCATGAACCTCAAGGACT-3' (sense)
5'-CACCATGGCAACATATTCC-3' (antisense)

Set 4: 5'-GCCAAAGAGATTCAAGATGATTGGAAGTATGTTGCCATGG-3' (sense)
5'-TCTATGTGTCATCTCTGGCCATCAAGGGTTGCAG-3' (antisense)

α4 nAChR primers

Set 1: 5'-TGCGTGCGCCATGGAGCTAGGGGGC-3' (sense)
5'-CGTACGTCCAGGAGCCGAACTTCATG-3' (antisense)

Set 2: 5'-ACGGRMGGGTGCAGTGGA-3' (sense)
5'-CTTCTGGCCMGAGCCWG-3' (antisense)

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Set 3: 5’-CGGCCCTCCGTGGTCAAGGACAACT-3’ (sense)

5’-TCCTAGATCATRCCAGCCA-3’ (antisense)

β2 nAChR primers

Set 1: 5’-CGGCTTCAGCACCACGGACAGCGCCCCACC-3’ (sense)

5’-CCGAGACTCGACCACTGACATGTCGAGTACC-3’ (antisense)

Set 2: 5’-ACKGAYACAGAGGAGCGG-3’ (sense)

5’-GAAGATAAGGTTACGRCACC-3’ (antisense)

Set 3: 5’-TCACMTGGAAGCCTGARGA-3’ (sense)

5’-GGTAGCAGTGGTCGCACA-3’ (antisense)

Set 4: 5’-GCGGCGAGAAGATGACGCTGTGCATCTCCG-3’ (sense)

5’-GGTAGCAGTGGTCGCACA-3’ (antisense)

Full-length cDNA was prepared using gene splicing by overlap extension and PCR amplification; resultant cDNA was confirmed by sequencing. These clones contained minimal or no 5’- or 3’-UTR sequence. cDNAs were subcloned into mammalian expression vectors by Eco RI digestion of the plasmids in the pCR2.1-TOPO vector, cDNA fragment isolation from 1% agarose gels, and subsequent ligation into the Eco RI site of either pcDNA3.1(-)Hygro (for the α4 and α3 subunits) or pcDNA3.1(-) (for the β2 subunit). Capped cRNA was prepared using mMessage mMachine (Ambion, Austin, TX) transcription via the T7 promoter in the pcDNA 3.1 vector.

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Expression of nAChR in *Xenopus laevis* oocytes

Female *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, Wisconsin) and were maintained and treated using standard protocols approved by Abbott's Institutional Animal Care and Use Committee. The preparation of *Xenopus laevis* oocytes, injection with cDNA or cRNA prepared by standard techniques, and measurement of nAChR responses using two-electrode voltage-clamp followed procedures similar to those described previously (Briggs et al., 1995). Briefly, ovaries were removed surgically from a *Xenopus laevis* frog under tricaine anesthesia (0.28% in deionized water) and oocytes were prepared following incubation for 1-2 hours at room temperature in collagenase (Sigma type 1A, 2 mg/ml) in low-Ca^{2+} Barth's solution (pH 7.55) containing 87.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM sodium N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer ("Na-HEPES") and 100 µg/ml gentamicin. Oocytes were maintained, before and after injection, at 17-18°C in normal Barth's solution (pH 7.55) containing 90 mM NaCl, 1 mM KCl, 0.66 mM $NaNO_3$, 0.74 mM $CaCl_2$, 0.82 mM $MgCl_2$, 2.4 mM $NaHCO_3$, 2.5 mM sodium pyruvate, 10 mM Na-HEPES buffer, and 100 µg/ml gentamicin. Glass Petri dishes were used to avoid any potential interference with nAChR function by substances found in some plastics (Papke et al., 1994).

For expression of nAChR, oocytes were injected within 24 hours of their preparation and were used 2-7 days after injection. Each oocyte was injected with either 40-50 nl nAChR RNA or 10-15 nl nAChR DNA. The total concentration of RNA or DNA was approximately 1 µg/µl determined spectrophotometrically. Injections were conducted using like-preparations only, e.g. RNA with RNA or DNA with DNA. Results were similar with either RNA or DNA, but RNA

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was used preferentially in studies with varied message ratios in order to avoid transcription variance.

For measuring functional nAChR responses, oocytes were transferred to room-temperature OR-2 solution (pH 7.4) containing 90 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM Na-HEPES buffer, and 0.5 μM atropine to block endogenous muscarinic receptors. In some experiments, $CaCl_2$ was replaced by $BaCl_2$ in order to prevent secondary activation of a Ca^{2+}-dependent Cl^- current. Compounds were applied and responses were measured at -60 mV cell potential in the POETs apparatus, a computer –controlled robotic device that controls compound delivery, electrophysiological response recording, and data storage and measurement in a searchable database (Trumbull et al., 2003). The device operates six oocyte-containing chambers, applies compounds using a robotic Gilson pipettor (typically, 4 ml/min for 4 seconds followed by 3-5 minutes wash by perfusion), records responses under two-electrode voltage-clamp using Axon Instruments Geneclamp 500 amplifiers, National Instruments A/D system and an IBM-compatible computer. Custom software was used to schedule compound application to the oocytes at user-defined intervals (typically 3-5 minutes), store the recordings in a searchable database, retrieve the responses, quantify the responses by peak amplitude or integral, and perform curve fitting or export the data to other software for further analysis. For the data presented here, concentration-response parameters were determined using the nonlinear curve-fitting in Graphpad Prism and the built-in variable slope sigmoidal curve (Hill equation) or a biphasic version that was the sum of two independent Hill equations. Typically, the concentration-response parameters for curve fitting were not constrained except that the bottom of the curve was set equal to 0; exceptions are noted.

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In each oocyte, responses to test compound were normalized to the maximal response to acetylcholine (100 μM or 1 mM as indicated, depending upon the nAChR), and the stability of responses during testing was monitored by applying acetylcholine at regular intervals during the experiment. Agonist responses typically were measured as the compound-induced peak (maximal) inward current relative to the baseline holding current. In some experiments, the response integral ("area under the curve") also was measured, with the beginning and end of the integration period defined by the beginning and end of the activation of the Gilson syringe pump used to apply compound. Similar concentration-response parameters were obtained by integral or peak amplitude.

Mouse brain synaptosome rubidium flux

To assess agonist potency and efficacy at native α4β2 nAChR, DHβE-sensitive stimulation of $^{86}Rb^{+}$ efflux from mouse thalamic synaptosomes was determined as described by Marks *et al.* (2004; 1999). C57BL/6J mice were bred at the Institute for Behavioral Genetics (University of Colorado, Boulder, CO) and were treated as approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder. The crude synaptosomal fraction was prepared by hand homogenization (Teflon-glass tissue grinder) in 10 volumes ice-cold 0.32 M sucrose with 5 mM HEPES buffer (pH 7.5). The homogenate was centrifuged at 500g for 10 minutes to pellet nuclei and heavy debris (P1) and the supernatant subsequently was centrifuged at 12,000g for 20 minutes to yield the synaptosomal pellet (P2). To load the synaptosomes with $^{86}Rb^{+}$, the P2 was resuspended in uptake buffer (140 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1

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mM $MgSO_4$, 20 mM glucose and 25 mM Na-HEPES buffer, pH 7.5) and incubated with 4 μCi $^{86}RbCl$ for 30 minutes in a final volume of 35 μl. Uptake was terminated by filtration onto a glass fiber filter (Gelman type AE, 6 mm diameter) and two 0.5 ml washes with uptake buffer. For experimental measurements, the loaded filter was transferred to a polypropylene platform and perfused at 2.5 ml/min with buffer containing 135 mM NaCl, 5 mM CsCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, 25 mM Na-HEPES buffer (pH 7.5), 50 nM tetrodotoxin, 1 μM atropine, and 0.1% bovine serum albumin fraction V. Compounds were applied by filling a 200 μl loop with appropriate solution and diverting perfusion buffer through the loop by means of a 4-way rotary Teflon injection valve. Efflux of $^{86}Rb^+$ was detected continuously by pumping perfusate through a 200 μl flow-through Cherenkov cell in a β-RAM Radioactivity HPLC Detector (IN/US Systems inc., Tampa, FL).

Total agonist-stimulated responses were calculated as the increase in signal above the basal efflux rate, which was calculated by a non-linear least squares fit of the data before and after the peak response (Marks et al., 2004; Marks et al., 1999). Responses were normalized by dividing the agonist-stimulated response by the basal efflux. Each experiment also included samples stimulated with 10 μM nicotine to facilitate comparison of results between experiments.

Materials

Acetylcholine, atropine, bovine serum albumin, collagenase Type IA, dihydro-β-erythroidine, 17β-estradiol, gentamicin, mecamylamine, methyllycaconitine, (-)-nicotine tartrate, and *d*-tubocurarine were purchased from Sigma Chemical Company (St. Louis, MO). Chlorisondamine

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was purchased from Tocris (Ellisville, MO). HEPES and sucrose were from Boehringer-Ingelheim (Indianapolis, IN). CsCl and Budget Solve scintillation fluid were from Research Products International (Mt. Prospect, IL). Carrier-free ^{86}RbCl was from DuPont-NEN (Boston, MA). A-163554 ((R)-2-chloro-3-(5,5-dimethyl-hexa-1,3-dienyl)-5-(pyrrolidin-2-ylmethoxy)pyridine), A-162035 ((R)-2-chloro-3-phenyl-5-(pyrrolidin-2-ylmethoxy)-pyridine) and A-168939 ((R)-5-chloro-6-(2-pyridin-4-yl-vinyl)-2-pyrrolidin-2-yl-furo[3,2-b]pyridine) were synthesized at Abbott Laboratories as described by Lin *et al.* (2001).

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Results

In cloning ferret α4 and β2 nAChR, two approaches were used, one using primers designed to encompass the coding region with minimal 3' and 5' extension, and the other using a cDNA library with oligonucleotide probes directed towards the coding regions. Because the latter approach is based upon hybridization to long, potentially full-length cDNA derived from mRNA, it permits isolation of cDNAs containing untranslated regions (UTR). Indeed, α4 and β2 messages with relatively long 3' and 5' UTR were isolated by the cDNA library screening. The relative sizes of the ferret α4 and β2 nAChR UTR segments are diagrammed in **Figure 1.** The **Supplemental Information** shows ferret α3, α4 and β2 nAChR amino acid sequences and ferret α4 and β2 nAChR UTR nucleotide sequences aligned with corresponding human and rat sequences.

Expression of high- and low-sensitivity α4β2 nAChR

Oocytes injected with RNA or DNA derived from these clones expressed functional α4β2 nAChRs, but with different results depending upon whether or not the messages contained UTR sequences. In the following, "α4(u)" refers to α4 coding sequence with 5' and 3' UTR; likewise, "β2(u)" refers to β2 coding sequence with 5' and 3' UTR.

Oocytes injected with ferret α4 and β2 (1:1 ratio) lacking UTR expressed typical acetylcholine-gated currents and a biphasic concentration-response relationship as reported previously using human and rat α4β2 (Nelson et al., 2003; Buisson and Bertrand, 2001; Chavez-Noriega et al.,

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2000; Zwart and Vijverberg, 1998). In contrast, when α4(u) and β2(u) were used, the concentration-response relationship was monophasic with an EC_{50} value similar to the high-sensitivity portion of the biphasic relationship seen using messages without the UTR. Concentration-response relationships for acetylcholine are shown in **Figure 2**, and extracted parameters are given in **Table 1**. In view of the unexpected results with the α4(u)β2(u) combination, the initial measurements were repeated in thirty oocytes from three donor *Xenopus laevis* with similar results from each cell.

Zwart and Vijverberg (1998) reported that increasing the proportion of β2 message to an α4:β2 ratio of 1:9 could lead to the appearance of a biphasic concentration-response curve with expression of a higher-sensitivity component. Reasoning that the effect we observed may result from higher levels of β2 protein due to increased translation of β2 due to the presence of UTR, we attempted to generate monophasic high-sensitivity acetylcholine concentration curves by adjusting the α4:β2 ratio using messages without UTR. Decreasing the α4:β2 ratio to as much as 1:120 increased the high sensitivity proportion (**Figure 3** and **Table 1**), however, the acetylcholine concentration-response curves remained biphasic. Thus, we were unable express exclusively monophasic high-sensitivity ferret α4β2 using messages lacking UTR.

To determine whether α4 UTR or β2 UTR was required for exclusive expression of the high-sensitivity α4β2 subform, α4 with or without UTR was combined with β2 with or without UTR. Using 1:1 ratios, UTR in both α4 and β2 appeared to be required (**Figure 4**) because the low-sensitivity subform clearly was expressed when either α4 without UTR or β2 without UTR was used. However, the β2 with UTR appeared to have the greater effect and could increase the

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expression of the high-sensitivity α4β2 subform even when α4 lacked UTR (**Figure 4C** and **Table 1**). Consistent with this, in further experiments it was found that high-sensitivity α4β2 could be exclusively expressed using α4 lacking UTR plus β2 with UTR in a ratio of 1:5::α4:β2(u) (**Figure 5**).

Expression of high- and low-sensitivity α3β2 nAChR

The above observations suggested that β2(u) could regulate the form of α4β2 nAChR expressed in oocytes. To determine whether this effect may generalize to other β2-containing nAChR, ferret α3 was combined with β2 and β2(u) in ratios ranging from 1:1 to 1:20::α3:β2. The acetylcholine-concentration-response curve for α3β2 1:1 could be fit with a biphasic curve and EC_{50}s of 25 and 450 μM (**Figure 6 and Table 2**). Using β2(u), however, a lower EC_{50} (3-9 μM) component appeared, predominated at an α3:β2(u) message ratio of 1:10, and was exclusively expressed at an α3:β2(u) message ratio of 1:20. Without β2-UTR, however, exclusive expression of the high-sensitivity α3β2 subform could not be achieved at a message ratio up to 1:20. Thus, β2(u) appeared to regulate expression of higher-sensitivity forms of α3β2 as well as α4β2.

Antagonist potency at α4β2 subforms

It remains unclear whether native α4β2 nAChR are better represented by the higher-sensitivity form, the lower sensitivity form, or whether both forms may be expressed and regulated

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differentially according to cell type or maturation. However, the α4(u) and β2(u) clones represent sequences that, because they contain partial or full UTR, are closer to the native mRNA that would be expressed in brain than are the clones without UTR. Thus, it was of interest to explore the pharmacology of the high- and low-sensitivity α4β2 nAChR with the aim of uncovering selective tools that could be used to elucidate the properties and physiological roles of the receptors.

Five antagonists were evaluated for their effects on high- and low-sensitivity forms of α4β2. This was performed using receptors expressed from α4(u)β2(u) to generate the high-sensitivity form alone, and from α4β2 without UTR to generate mixed high- and low-sensitivity receptors; we were not able to express the low-sensitivity form alone. With both α4(u)β2(u) and α4β2, antagonist IC_{50} values were measured against two concentrations of acetylcholine, 2 μM (near the high-sensitivity EC_{50}) and 200 μM (near the low-sensitivity EC_{50}). In the mixed-sensitivity α4β2 population, most (~97%) of the response to 2 μM acetylcholine should have been from the high-sensitivity α4β2 subform, while for 200 μM acetylcholine most (~81%) of the response should have been from the low-sensitivity α4β2 subform based upon concentration-response parameters shown in **Table 1**. The antagonist concentration-inhibition curves are shown in **Figures 7** and **8** and IC_{50} values are in **Table 3**.

Neither dihydro-β-erythroidine (DHβE) nor methyllycaconitine distinguished between the high- and low-sensitivity forms (**Figure 7**). IC_{50} values were 3-6 nM for DHβE and 40-135 nM for methyllycaconitine under all conditions. In contrast, chlorisondamine, and to some extent mecamylamine and *d*-tubocurarine, appeared to be selective for the low sensitivity form (**Figure**

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8). Using 200 μM acetylcholine and the mixed-sensitivity α4β2, the IC_{50} values were 0.2 μM for mecamylamine, 0.9 μM for *d*-tubocurarine, and 0.2 μM for chlorisondamine. Using the isolated high sensitivity form, α4(u)β2(u), and 2 μM acetylcholine, IC_{50} values were 8-, 5-, and 100-fold higher for mecamylamine, *d*-tubocurarine and chlorisondamine, respectively.

Modulation of α4β2 subforms by estradiol

17β-estradiol is a neuroactive steroid which has been found to potentiate human α4β2 while inhibiting other nAChR (Curtis et al., 2002; Paradiso et al., 2001; Nakazawa and Ohno, 2001). Estradiol clearly potentiated the acetylcholine response at the high-sensitivity ferret α4(u)β2(u), as shown in **Figure 9**. In the mixed sensitivity population, however, the potentiation was weaker. It was not clear whether this was due to a selective potentiation of the high-sensitivity subform, or to a mixture of effects at both subforms.

Agonist efficacy at α4β2 subforms

In rat brain, α4β2 comprise the majority of the high-affinity binding sites for (-)-nicotine (Flores et al., 1992; Whiting et al., 1991). However, in the mixed-sensitivity population generated from α4β2 messages lacking UTR, the apparent potency and efficacy values for (-)-nicotine were similar to those for acetylcholine (**Figure 10**). In the high-sensitivity populations generated from α4 and β2 messages containing UTR, or α4 message lacking UTR plus β2 message containing UTR (1:5 message ratio), (-)-nicotine was as potent as in the mixed-sensitivity population, but its apparent efficacy was only 24% relative to acetylcholine.

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In contrast, analogues of A-84543 (3-[2-((S)-pyrrolidinyl)methoxypyridine) (Lin et al., 2001) were found to be highly selective for the high-sensitivity α4β2 subform, based upon efficacy determinations using α4(u)β2(u) and α4β2. For example, (R)-2-chloro-3-(5,5-dimethyl-hexa-1,3-dienyl)-5-(pyrrolidin-2-ylmethoxy)pyridine (A-163554) was highly efficacious at the ferret high-sensitivity α4β2 expressed from UTR-containing α4(u)β2(u) but appeared as if it were a partial agonist in the mixed high- and low-sensitivity populations expressed from α4β2 lacking UTR (**Figure 11**). Similarly, (R)-2-chloro-3-phenyl-5-(pyrrolidin-2-ylmethoxy)-pyridine (A-162035, **Figure 12**) and (R)-5-chloro-6-(2-pyridin-4-yl-vinyl)-2-pyrrolidin-2-yl-furo[3,2-b]pyridine (A-168939, **Figure 13**) were, in comparison to acetylcholine, full agonists at α4(u)β2(u) but seemingly partial agonists in the mixed-sensitivity α4β2 population. These compounds appear to selectively activate the high-sensitivity α4β2 response, thus producing an apparent partial response from oocytes expressing low- as well as high-sensitivity α4β2.

A-163554 and A-168939 were somewhat less efficacious at α4β2 than anticipated from their efficacy at α4(u)β2(u) and assumption of 15% high-sensitivity subform in the mixed-sensitivity α4β2 population. This may be due to functional differences between high-sensitivity subforms from α4(u)β2(u) compared to α4β2, or to variance in the relative amount of the high-sensitivity subform expressed from α4β2. A-162035 appeared more efficacious than the other analogues at α4β2, probably because of some activity at low-sensitivity as well as high-sensitivity α4β2.

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Agonist efficacy at native α4β2

A-162035 and A-168939 were used to test whether receptors similar to the high-sensitivity α4β2 subform could be expressed in brain. A-162035 (**Figure 14A**) and A-168939 (**Figure 14B**) each stimulated α4β2-mediated $^{86}Rb^{+}$ flux in mouse thalamic synaptosomes. The EC_{50} values for $^{86}Rb^{+}$ flux (see figure legend) were remarkably similar to the EC_{50} values determined using ferret α4(u)β2(u), despite the differences in species and assay types. Furthermore, maximal responses to A-162035 and A-168939 were nearly as large as the response to 10 μM (-)-nicotine which has been shown to be selective for the high-sensitivity α4β2 nAChR response in mouse thalamus (Marks et al., 2004; Marks et al., 1999). **Figure 14C** also shows the thalamic synaptosome response to 10 μM (-)-nicotine in relation to the biphasic acetylcholine concentration-response relationship. Responses to 10-100 μM A-162035 and A-168939 were essentially completely blocked by 2 μM DHβE, which also has been shown to be selective for the high-sensitivity α4β2 nAChR in this assay.

In mouse brain synaptosomes, A-168939 appeared to be slightly more efficacious than A-162035 while the reverse was found using ferret α4(u)β2(u) expressed in oocytes. Nevertheless, the synaptosome data for A-162035 and A-168939 agree well with the oocyte α4(u)β2(u) data in contrast to the mixed-sensitivity α4β2 data. Overall, the results are consistent with the idea that the high-sensitivity α4β2 subform is expressed in brain and that the agonists A-162035 and A-168939 selectively activate that receptor.

Higher concentrations of A-162035 (≥ 3 μM) and A-168939 (≥ 10 μM) appeared to inhibit the synaptosomal response to the same compounds (**Figure 14**), possibly because of nAChR channel block or desensitization. A similar effect, at somewhat higher concentrations, was observed with ferret α4β2 expressed in oocytes (**Figures 12 and 13**). High concentrations of acetylcholine and nicotine also can produce an inhibitory effect (**Figures 2-10**). The mechanism of this inhibition was not investigated.

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Discussion

The main findings in this study are that: (a) ferret α4β2 nAChR could be expressed exclusively in the high-sensitivity form only from UTR-containing message; (b) the principal determinant appears to be in the β2 UTR although α4 UTR also may contribute; (c) a high sensitivity form of α3β2 also could be exclusively expressed with UTR-containing β2; (d) high- and low-sensitivity α4β2 could be distinguished pharmacologically by certain antagonists and agonists as well as by the potency of the neurotransmitter acetylcholine; (e) agonists selective for the high-sensitivity α4β2 subform were active at native α4β2 in mouse brain as well as at recombinant ferret α4β2.

It has been reported that the proportion of high-sensitivity α4β2 could be increased by increasing the amount of β2 message (Zwart and Vijverberg, 1998) or by prolonged exposure to low concentrations of nicotine or reduced temperature (Nelson et al., 2003; Buisson and Bertrand, 2001). Zhou *et al.* (2003) also revealed biphasic concentration-response curves and monophasic high-sensitivity concentration-response curves for acetylcholine depending upon the α4-β2 concatamer arrangement or the addition of free β2 message. These studies have suggested that high- and low-sensitivity components may correspond to $\alpha4_{(2)}\beta2_{(3)}$ and $\alpha4_{(3)}\beta2_{(2)}$ pentamers, respectively.

Using ferret messages, increasing the relative amount of β2 message appeared to increase the proportion of high-sensitivity α4β2, similar to previous reports with α4β2 from other species. Zwart and Vijverberg (1998) also observed mixed high- and low-sensitivity α4β2, even with the 1:9 message ratio. However, *exclusive* expression of the high-sensitivity α4β2 subform (or the

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high-sensitivity α3β2 subform) could be achieved when using ferret β2 message containing UTR, but not when using messages lacking UTR. It is assumed that the same α4 and β2 proteins are expressed with or without UTR. High-sensitivity ferret α4β2 expression may be particularly dependent upon the presence of UTR for message stability or protein translation, and at very low α4:β2 ratios without UTR the small amount of α4 may limit the ability to detect functional α4β2 expression. Short UTR segments in the human messages (Nelson et al., 2003; Zhou et al., 2003) and possibly rat messages (Zwart and Vijverberg, 1998) used in prior reports also may have influenced high-sensitivity α4β2 expression; this remains to be investigated. Additionally, it should be noted that the β2 TM3-TM4 cytoplasmic loop is shorter in ferret β2 than in human and rat β2, largely due to two sequences of amino acids, one of 8 amino acids located 38 residues upstream from TM4 and the other of 13 amino acids located 15 residues further upstream. Potentially, α4β2 or α3β2 assembly could be affected by the shorter loop. However, next to TM3 and TM4 the critical "proximal" amino acids of the cytoplasmic loop (Kuo et al., 2005) are identical in ferret, human and rat.

The ferret α4 UTR also appeared to have an effect on exclusive expression of the high-sensitivity form. Interestingly, the 5' α4 UTR contains an open reading frame (ORF) that appears to be conserved among ferret, rat and human (**Supplemental Information**). Examples of an upstream ORF affecting downstream translation are known (Morris and Geballe, 2000). However, there is no direct evidence that the α4 5' ORF affects coding sequence translation or is itself translated.

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For α3β2, a wide range of acetylcholine EC_{50} values have been reported, from 1.2 to 443 μM (Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997; Gerzanich et al., 1995) and Covernton and Connolly (2000) suggested a biphasic α3β2 concentration-response. Using ferret β2 with UTR, we demonstrated that α3β2 as well as α4β2 indeed could exhibit a biphasic concentration-response relationship for acetylcholine. Furthermore, the high-sensitivity α3β2 subform could be exclusively expressed using a 1:20 ratio of α3:β2(u). To our knowledge, this is the first report that decreasing α3:β2 message ratio influences α3β2 sensitivity to acetylcholine, and the first exclusive expression of the high-sensitivity subform.

In many studies with recombinant α4β2 nAChR, higher EC_{50} forms appear to predominate (Nelson et al., 2003; Houlihan et al., 2001; Chavez-Noriega et al., 2000; Gopalakrishnan et al., 1996), while predominant low EC_{50} values are observed in others (Kuryatov et al., 1997; Buisson et al., 1996; Bertrand et al., 1990). In CNS, α4β2 nAChR demonstrate low EC_{50} corresponding to high-sensitivity α4β2 (Marszalec et al., 1999; Marks et al., 1999; Alkondon and Albuquerque, 1995; Alkondon and Albuquerque, 1993; Marks et al., 1993). Clearly, such variances raise questions regarding the extension of recombinant nAChR pharmacology to native nAChR.

To identify compounds that may be useful in evaluating the physiological roles of high- and low-sensitivity α4β2, several antagonists and agonists were evaluated for selectivity. These experiments utilized α4(u)β2(u) to express exclusively the high-sensitivity subform, and α4β2 to express a mixture of high- and low-sensitivity subforms. While the antagonists DHβE and methyllycaconitine were not selective between α4β2 subforms, chlorisondamine, mecamylamine and *d*-tubocurarine were somewhat selective for the low-sensitivity α4β2 subform. Our results

with *d*-tubocurarine were generally similar to those of Zwart and Vijverberg (Zwart and Vijverberg, 1998) using another species' α4β2. Both studies found low IC_{50} (0.5-1 μM) and low Hill coefficient (0.71-0.77) for 1:1 α4β2 and high concentrations of acetylcholine (200 or 300 μM), and both found similar values (2-5 μM IC_{50}s, 0.67-0.78 Hill coefficients) for high sensitivity α4β2 and lower concentrations of acetylcholine (2 or 10 μM). With 1:9 α4β2 and 300 μM acetylcholine, Zwart and Vijverberg (1998) observed a biphasic concentration-inhibition curve, although it is not clear to what extent this was due to *d*-tubocurarine properties or the mixture of low- and high-sensitivity α4β2 obtained with the 1:9 ratio. In our experiments with α4(u)β2(u) and 200 μM acetylcholine or 1:1 α4β2 and 2 μM acetylcholine, we observed high IC_{50} values (50-100 μM) and low Hill coefficients (0.39-0.41) possibly reflecting an unresolved combination of low and high potencies for *d*-tubocurarine. The different potencies of *d*-tubocurarine at α4β2 may reflect differences between high- and low sensitivity α4β2 receptors, differences between the two binding sites in each receptor, or different mechanisms of inhibition such as binding site displacement and channel block.

In addition to antagonists selective for low-sensitivity α4β2, agonists displaying efficacy selective for high-sensitivity α4β2 could be identified. Analogs of A-84543 (Lin et al., 2001) appeared to activate predominantly high-sensitivity α4β2. A-163554, A-162035 and A-168939 were full agonists at the high-sensitivity α4(u)β2(u) subform. In contrast, these compounds had the appearance of partial agonists in the mixed-sensitivity α4β2 population expressed from message lacking UTR, to an extent consistent with high efficacy at the high-sensitivity component and low efficacy at the low-sensitivity component.

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To determine whether such compounds could activate native α4β2, the effect on $^{86}Rb^{+}$ flux in mouse brain thalamic synaptosomes was measured under conditions selective for the α4β2 component. A-162035 and A-168939 stimulated $^{86}Rb^{+}$ flux to an extent nearly similar to that of 10 μM nicotine, which has been shown to produce a near-maximal α4β2 effect in this assay (Marks et al., 2004; Marks et al., 1999). Indeed, the EC_{50} values for these compounds in mouse brain were similar to the values determined using high-sensitivity α4(u)β2(u) expressed in *Xenopus* oocytes. Further, thalamic responses to A-162035 and A-168939 were blocked by the α4β2 antagonist DHβE. These observations support the idea that high-sensitivity α4β2 represents a native α4β2 nAChR.

A simple assumption is that the mixed-sensitivity α4β2 responses resulted from expression of different α4β2 receptors (e.g. $\alpha4_{(3)}\beta2_{(2)}$ and $\alpha4_{(2)}\beta2_{(3)}$) with the high-sensitivity component ($\alpha4_{(2)}\beta3_{(3)}$) corresponding to the receptor expressed from UTR-containing α4 and β2 messages or low α4:β2 ratios. Biphasic concentration-response curves were fit by the sum of two Hill equations, assuming independent activation of the two components. Most data were consistent with these assumptions. However, some apparent discrepancies were noted. Chlorisondamine was less potent against α4(u)β2(u) than α4β2 stimulated by 2 μM acetylcholine even though responses were expected to be predominantly (≥ 97%) from the receptor with high-sensitivity to acetylcholine in both measurements. Nicotine was a partial agonist (24%) at α4(u)β2(u), yet it appeared to be essentially a full agonist at the high-sensitivity component of ferret mixed-sensitivity α4β2 expressed in oocytes and at the high-sensitivity component in mouse thalamic synaptosomes. The explanation is not known, but it is possible that high- and low-sensitivity components result from differences in the binding sites within the nAChR pentamer (e.g. α-α

versus α-β), non-independent α-β dimer function conditioned by the fifth subunit in the pentamer, or perhaps larger-scale interactions in receptor clusters.

The UTR-containing mRNAs that facilitated expression of high-sensitivity α4β2 and α3β2 represent naturally-expressed messages. UTRs can regulate expression at the mRNA and/or protein levels. Within some UTRs are sequences that can interact with regulatory proteins, RNA sequences, or other molecules and thereby provide means for regulating the expression of the encoded protein (Wilusz and Wilusz, 2004; Mazumder et al., 2003; Morris and Geballe, 2000). Through such processes, the expression of high- and low-sensitivity nAChR subforms may be regulated in neurons, possibly developmentally, according to cell type, or in response to various extracellular messengers. Such regulatory processes potentially could impact a variety of nAChR physiological and pharmacological actions, including nicotine dependence, antinociception, and cognitive function.

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Figure Legends

Figure 1. Ferret α4 and β2 UTR. The sizes of the 5' UTR (top-down diagonal) and 3' UTR (bottom-up diagonal) segments of ferret α4 and β2 cloned from the cDNA library are diagrammed relative to the length of the coding sequences. The β2 3' UTR (1267 nucleotides) was nearly as long as the coding region (1437 nucleotides). Complete sequences are shown in **Supplemental Information**.

Figure 2. Concentration-response curves for acetylcholine at ferret α4β2 nAChR expressed from messages with and without UTR. Ferret nAChR subunits were expressed in *Xenopus laevis* oocytes using standard techniques and responses to acetylcholine applied for 5 seconds were obtained at –60 mV and measured at peak amplitude relative to the baseline holding current. In each oocyte, responses to various concentrations of acetylcholine were normalized to reference control responses in order to account for cell to cell variance in level of receptor expression. The reference control, designed to elicit a maximal response (100 μM acetylcholine for high-sensitivity α4β2 from UTR-containing messages and 1 mM acetylcholine for other nAChR), was contained in a solution separate from the test solutions, and was applied at regular intervals during the experiment in order to detect fluctuations in responsiveness. (**A)** shows data combined from thirty oocytes (three separate preparations) injected with UTR-containing α4 (α4(u)) and β2 (β2(u)) nAChR messages in approximately equal amounts (1:1 ratio). The curve shows a Hill equation fit to the combined data with an EC_{50} value of 2.2 μM (1.9-2.6 μM c.i.), Hill slope of 1.05 ± 0.06 and plateau of 105 ± 0.8 %. Curves fit to individual data from each of twenty-eight oocytes provided similar results; in the other two oocytes, data

were insufficient for curve fitting. **B.** Data are from seven oocytes injected with a 1:1 ratio α4 and β2 nAChR messages lacking UTR. The fitted curve is the sum of two independent Hill equations with EC_{50} values of 0.54 μM (0.086-3.41 μM c.i.) and 114 μM (90-145 μM c.i.), Hill slopes of 1.01 ± 0.35 and 1.39 ± 0.23 and plateaus of 13 ± 4 % and 84 ± 6 %. Data are shown as mean ± SEM or mean ± 95% confidence interval (c.i.) for EC_{50} values.

Figure 3. Inability to isolate high- and low-sensitivity components by adjusting ratio of messages lacking UTR. Oocytes were injected with α4 and β2 subunit messages in various ratios according to nucleotide content. Volume and total nucleotide content were similar. Relative amounts injected were **(A)** 9 α4 to 1 β2, **(B)** 1 α4 to 1 β2, **(C)** 1 α4 to 9 β2, **(D)** 1 α4 to 20 β2, **(E)** 1 α4 to 60 β2 and **(F)** 1 α4 to 120 β2. Data for **(B)** are the same as in **Figure 2B** and are reproduced here to facilitate comparison with other α4:β2 ratios. Concentration-response parameters, determined as in Figure 2, are provided in Table 1.

Figure 4. Both α4 and β2 UTR contribute to exclusive expression of the high-sensitivity α4β2 nAChR. Oocytes were injected with equal amounts of α4 subunit message without or with 3'- and 5'-UTR plus β2 subunit message without or with 3'- and 5'-UTR. Data are shown for **(A)** α4β2 nAChR from α4 and β2 messages both lacking UTR, **(B)** α4(u)β2 nAChR from α4 message containing 3'- and 5'-UTR and β2 message lacking UTR, **(C)** α4β2(u) nAChR from α4 message lacking UTR and β2 message containing 3'- and 5'-UTR, and **(D)** α4(u)β2(u) nAChR from α4 and β2 messages both containing 3'- and 5'-UTR. Concentration-response parameters, determined as in Figure 2, are provided in Table 1.

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Figure 5. Increased proportion of β2 message containing UTR eliminates the low-sensitivity component. Oocytes were injected with α4 and β2 nAChR subunit messages both lacking UTR (open symbols) or α4 nAChR subunit message lacking UTR plus β2 nAChR subunit message containing 3'- and 5'-UTR in a 1:5 nucleotide content ratio (closed symbols). Exclusive expression of the high-sensitivity component was obtained with an excess of UTR-containing β2 subunit relative to α4 subunit. Data are from four oocytes of each type measured on the same day and concentration-response parameters are provided in Table 1.

Figure 6. Expression of high-sensitivity α3β2 nAChR using UTR-containing message. Oocytes were injected with α3 subunit message lacking UTR plus β2 subunit message either lacking UTR (open symbols) or containing 3'- and 5'-UTR (closed symbols). The α3:β2 nucleotide content ratios were **(A)** 1:1, **(B)** 1:5, **(C)** 1:10 and **(D)** 1:20. Concentration-response parameters are provided in Table 2.

Figure 7. Antagonists dihydro-β-erythroidine and methyllycaconitine are not selective for high- versus low-sensitivity α4β2 nAChR. Concentration-inhibition data for dihydro-β-erythroidine (DHβE, parts **A & B**) and methyllycaconitine (MLA, parts **C & D**) were obtained in oocytes expressing a mixture of high- and low-sensitivity α4β2 nAChR (open symbols) and in oocytes expressing exclusively the high-sensitivity α4β2 nAChR from UTR-containing α4 and β2 messages (closed symbols). For both mixed-sensitivity and exclusively high-sensitivity nAChR, inhibition was measured against 2 μM acetylcholine **(**parts **A & C)** and 200 μM acetylcholine **(**parts **B & D)**. Concentration-inhibition parameters are provided in Table 3.

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Figure 8. Three antagonists appear selective for the low-sensitivity α4β2 component. Concentration-inhibition data for mecamylamine (parts **A & B**), *d*-tubocurarine (parts **C & D**) and chlorisondamine (parts **E & F**) were obtained as in Figure 8. Data are shown for oocytes expressing mixed-sensitivity α4β2 nAChR (open symbols) and high-sensitivity α4β2 nAChR from UTR-containing α4 and β2 messages (closed symbols). For both mixed- and high-sensitivity α4β2, the inhibition of responses to 2 μM acetylcholine **(A, C & E)** and 200 μM acetylcholine **(B, D & F)** was measured. Concentration-inhibition parameters are provided in Table 3.

Figure 9. Estradiol potentiation of α4β2 nAChR. Acetylcholine concentration-response data in the absence and presence of 10 μM 17β-estradiol were obtained from three oocytes expressing high-sensitivity α4β2 nAChR using UTR-containing messages and from three oocytes expressing mixed-sensitivity α4β2 nAChR using messages lacking UTR. The concentration of acetylcholine in the reference control was 100 μM for both mixed- and high-sensitivity α4β2 oocytes. **(A)** At high-sensitivity α4β2 nAChR, estradiol increased the maximal response by 64%. In the absence (open symbols) and presence (closed symbols) of estradiol, respectively, the acetylcholine EC_{50} values were 2.2 μM (1.8-2.6 μM c.i.) and 1.7 μM (1.0-2.7 μM c.i.), Hill slopes were 1.10 ± 0.10 and 1.10 ± 0.24 and plateaus were 102 ± 2 % and 166 ± 8 % relative to 100 μM acetylcholine in the absence of estradiol. **(B)** Estradiol also potentiated acetylcholine responses in the mixed-sensitivity population, but overall the effect was smaller than with high-sensitivity α4β2 alone. Concentration-response parameters were determined as for other data, except that Hill slopes were constrained to be shared between data with and without estradiol in

order to fit biphasic curves. For the high-sensitivity component, in the absence (open symbols) and presence (closed symbols) of estradiol, respectively, the acetylcholine EC_{50} values were 1.2 μM (0.59-2.6 μM c.i.) and 0.82 μM (0.31-2.2 μM c.i.), Hill slopes were 1.06 ± 0.18 and plateaus were 24 ± 3 % and 43 ± 8 % relative to control 100 μM acetylcholine in the absence of estradiol. For the low-sensitivity component in the absence and presence of estradiol, respectively, the acetylcholine EC_{50} values were 290 μM (210-400 μM c.i.) and 120 μM (77-190 μM c.i.), Hill slopes were 1.31 ± 0.13 and plateaus were 320 ± 30 % and 310 ± 40 % relative to control 100 μM acetylcholine in the absence of estradiol.

Figure 10. Effects of nicotine in the high- and mixed-sensitivity α4β2 populations.

Responses to (-)-nicotine were normalized to control 1 mM acetylcholine in each oocyte. In the mixed-sensitivity α4β2 population generated from messages lacking UTR (n=15), (-)-nicotine activated both high- and low-sensitivity components. The fitted curve shows EC_{50} values of 1.1 μM (0.52-2.5 μM c.i.) and 41 μM (31-54 μM c.i.), Hill slopes of 1.2 (constrained) and 2.3 ± 0.78 and plateaus of 31 ± 5 % and 63 ± 7 % relative to 1 mM acetylcholine. However, in the high-sensitivity populations generated using α4 and β2 messages both containing UTR (α4(u)β2(u), 1:1 ratio, n=4), the apparent efficacy of (-)-nicotine was less. The EC_{50} value was 0.49 μM (0.22-1.1 μM c.i.), the Hill slope was 1.16 ± 0.43 and the plateau was 24 ± 2 %. Similar results were obtained using α4 message lacking UTR and β2 message containing UTR (α4β2(u)::1:5, n=3), with which the (-)-nicotine EC_{50} value was 0.40 μM (0.24-0.66 μM c.i.), the Hill slope was 1.20 ± 0.30 and the plateau was 24 ± 1 %. Fitted curves for both high-sensitivity subforms are shown in the figure but are essentially overlapping.

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Figure 11. Selectivity of A-163554 for high-sensitivity α4β2. Responses are shown for A-163554 acting on the high-sensitivity receptor expressed from α4 and β2 messages containing UTR (α4(u)β2(u), n=3) and the mixed-sensitivity population expressed from messages lacking UTR (α4β2, n=3). As an additional control, full concentration-response relationships for acetylcholine were determined in the same three α4β2 oocytes exposed to A-163554. The fitted curves for A-163554 at α4(u)β2(u) show an EC_{50} value of 4.8 μM (3.1-7.3 μM c.i.), Hill slope of 1.34 ± 0.28 and plateau of 167 ± 12 % relative to 1 mM acetylcholine. Using mixed-sensitivity α4β2, the responses to A-163554 again were fitted well by a monophasic concentration-response curve, with an EC_{50} value of 0.74 μM (0.23-2.4 μM c.i.), Hill slope of 0.85 ± 0.30 and plateau of 15 ± 2 % relative to 1 mM acetylcholine. The same α4β2 oocytes demonstrated a biphasic concentration response to acetylcholine with EC_{50} values of 0.92 μM (0.39-2.2 μM c.i.) and 105 μM (92-119 μM c.i.), Hill slopes of 1.05 ± 0.26 and 1.35 ± 0.13, and plateaus of 19 ± 3 % and 85 ± 5 % relative to 1 mM acetylcholine.

Figure 12. Selectivity of A-162035 for high-sensitivity α4β2. Responses are shown for A-162035 acting on the high-sensitivity receptor expressed from α4 and β2 messages containing UTR (α4(u)β2(u), n=3) and the mixed-sensitivity population expressed from messages lacking UTR (α4β2, n=4). The fitted curves for A-162035 at α4(u)β2(u) show an EC_{50} value of 0.13 μM (0.11-0.16 μM c.i.), Hill slope of 1.49 ± 0.20 and plateau of 113 ± 3 % relative to 1 mM acetylcholine. Using α4β2 mixed-sensitivity receptors, A-162035 did not have a clear biphasic concentration-response relationship, but the low Hill slope suggested that the compound may act upon more than one receptor type. The fitted curve shown reflects an EC_{50} value of 4.6 μM

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(0.02-108 μM c.i.), Hill slope of 0.57 ± 0.35 and plateau of 41 ± 26 % relative to 1 mM acetylcholine.

Figure 13. Selectivity of A-168939 for high-sensitivity α4β2. Responses are shown for A-168939 acting on the high-sensitivity receptor expressed from α4 and β2 messages containing UTR (α4(u)β2(u), n=6) and the mixed-sensitivity population expressed from messages lacking UTR (α4β2, n=6). The fitted curve for A-168939 at α4(u)β2(u) shows an EC_{50} value of 0.71 μM (0.54-0.93 μM c.i.), Hill slope of 1.31 ± 0.18 and plateau of 86 ± 3 % relative to 1 mM acetylcholine. A-168939 had little effect at α4β2 mixed-sensitivity receptors. The fitted curve reflects an EC_{50} value of 7.5 μM (0.16-360 μM c.i.), Hill slope of 0.65 ± 0.40 and plateau of 8 ± 4 % relative to 1 mM acetylcholine. The low Hill slope may reflect activation of more than one receptor type, or simply the difficulty in resolving the concentration-response relationship for such small responses.

Figure 14. Activation of native α4β2 nAChR by A-162035 and A-168939. Mouse thalamic synaptosome Rb^+ flux was measured in response to A-162035, A-168939 and acetylcholine in the absence and presence of DHβE. Graphs also show the response to 10 μM (-)-nicotine (solid diamond) measured in each experiment as a positive control for high-sensitivity α4β2. **(A)** shows the concentration-response for A-162035 in the absence (solid triangles, n=4-5 each data point) and presence (open triangles, n=3) of 2 μM DHβE. The fitted curve for A-162035 shows an EC_{50} value of 0.12 μM (0.039-0.34 μM c.i.), Hill slope of 1.00 ± 0.33, and plateau of 8 ± 2 % relative to the maximal response. **(B)** shows the concentration-response for A-168939 in the absence (solid triangles, n=4-5 each data point) and presence (open triangles, n=3) of 2 μM

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DHβE. The fitted curve for A-168939 shows an EC_{50} value of 0.24 μM (0.11-0.50 μM c.i.), Hill slope of 1.00 ± 0.24, and plateau of 10 ± 1 % relative to the maximal response. **(C)** for comparison shows the concentration-response for acetylcholine in the absence (solid triangles, n=4) and presence (open triangles, n=4) of 2 μM DHβE. Note the change in ordinate and abscissa scales compared to (A) and (B). The concentration-response curve for acetylcholine was biphasic in the absence of DHβE. The fitted curve shows a high-sensitivity component with EC_{50} value of 0.77 μM (0.24-2.4 μM c.i.), Hill slope of 1.06 ± 0.40, and plateau of 18 ± 4 % and a low-sensitivity component with EC_{50} value of 57 μM (28-115 μM c.i.), Hill slope of 2.00 ± 0.97, and plateau of 16 ± 4 %.

Table 1. Isolation of a high-sensitivity α4β2 subform using UTR-containing messages expressed in oocytes.

Data for EC_{50} are shown as mean and 95% confidence interval. Data for the Hill coefficient (nH) are shown as mean ± SEM. For α4(u)β2(u) 1:1 and α4β2(u) 1:5, the data were fit by the monophasic Hill equation. Otherwise, the data were better fit by a biphasic curve representing the sum of two independent Hill equations; the proportion of the high affinity component was estimated from the plateau values of the two fitted components.

			acetylcholine high-sensitivity			acetylcholine low-sensitivity	
Receptor[a]	**Ratio α:β**	**N**	**EC_{50}, μM**	**nH**	**proportion**	**EC_{50}, μM**	**nH**
α4(u)β2(u)	1:1	30	2.2 (1.9-2.6)	1.05 ± 0.06	100%	--	--
α4β2	9:1	5	0.70 (0.4-1.2)	1.5 ± 0.3	15%	130 (80-190)	1.2 ± 0.1
α4β2	1:1	7	0.54 (0.09-3.4)	1.0 ± 0.4	14%	114 (90-145)	1.4 ± 0.2
α4β2	1:9	6	2.3 (0.41-13)	1.2 ± 0.3	35%	120 (56-270)	1.4 ± 0.8
α4β2	1:20	3	1.2 (0.05-30)	0.9 ± 0.7	32%	200 (71-540)	1.1 ± 0.6

			acetylcholine high-sensitivity			acetylcholine low-sensitivity	
Receptor[a]	**Ratio α:β**	**N**	**EC_{50}, μM**	**nH**	**proportion**	**EC_{50}, μM**	**nH**
α4β2	1:60	9	3.1 (1.1-8.4)	1.0 ± 0.3	38%	160 (120-210)	1.7 ± 0.3
α4β2	1:120	5	2.0 (0.41-9.5)	1.1 ± 0.5	46%	100 (50-210)	1.6 ± 0.8
α4β2	1:1	3	2.5 (0.22-29)	0.9 ± 0.5	24%	140 (110-180)	2.0 ± 0.4
α4(u)β2	1:1	4	4.0 (0.30-55)	1.1 ± 0.6	21%	200 (120-360)	1.5 ± 0.6
α4β2(u)	1:1	10	0.85 (0.16-4.6)	1.3 ± 1.1	39%	120 (45-350)	1.1 ± 0.6
α4(u)β2(u)	1:1	3	2.1 (2.0-2.2)	1.15 ± 0.02	100%	--	--
α4β2	1:1	4	1.5 (0.56-4.2)	1.1 ± 0.4	16%	180 (160-210)	1.5 ± 0.2
α4β2(u)	1:5	4	1.6 (1.2-2.3)	1.2 ± 0.2	100%	--	--

[a] Receptors were expressed from α4 and β2 messages lacking UTR and from other messages, designated α4(u) and β2(u), that contained the same coding regions plus 3' and 5' UTR segments.

Table 2. Potencies for acetylcholine at high-sensitivity and mixed- sensitivity ferret α3β2.

For α3β2(u) 1:10 and 1:20 ratios, the data for acetylcholine concentrations up to 1 mM were fit with a monophasic Hill equation. Other data were fit with a biphasic curve, as in Table 1. Hill coefficients for α3β2 1:20 were constrained in order to fir the data.

			acetylcholine high-sensitivity			acetylcholine low-sensitivity	
Receptor[a]	**Ratio α:β**	**N**	**EC_{50}, μM**	**nH**	**proportion**	**EC_{50}, μM**	**nH**
α3β2	1:1	15	25 (7-88)	1.2 ± 0.2	15%	450 (380-540)	1.3 ± 0.1
α3β2	1:20	6	23 (7-81)	1.2	34%	480 (200-1100)	1.2
α3β2(u)	1:1	13	8.1 (7.1-9.1)	0.89 ± 0.03	77%	1700 (1300-2100)	2.6 ± 0.3
α3β2(u)	1:5	3	2.8 (1.4-5.6)	1.5 ± 0.4	52%	88 (29-260)	1.1 ± 0.6
α3β2(u)	1:10	9	9.4 (6.6-13)	1.0 ± 0.2	100%	--	--
α3β2(u)	1:20	8	8.6 (6.0-12)	0.98 ± 0.14	100%	--	--

[a] Receptors were expressed from α3 message lacking UTR and from β2 messages lacking (β2) or containing (β2(u)) 3' and 5' UTR segments.

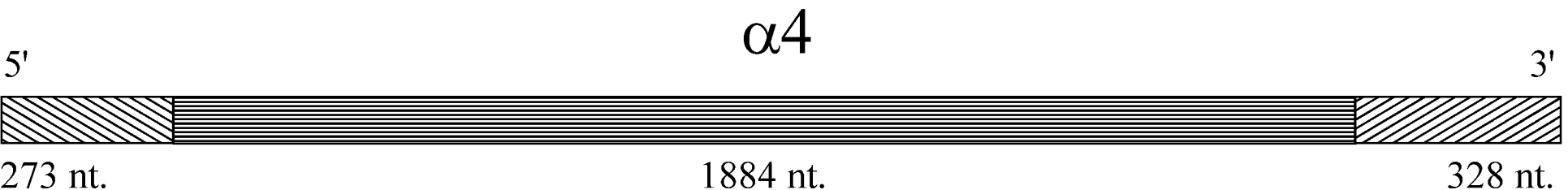
Table 3. Antagonist potencies at ferret α4β2 and at the high-sensitivity form expressed from UTR-containing α4(u) and β2(u).

Concentration-inhibition curves projected to 100% inhibition for methyllycaconitine (MLA), mecamylamine (Mec), *d*-tubocurarine (dTC) and chlorisondamine (CI), but not for dihydro-β-erythroidine (DHβE). For DHβE acting on α4β2 the projected maximal inhibition was 80 ± 3 % with 2 μM acetylcholine and 95 ± 4 % with 200 μM acetylcholine while these values at α4(u)β2(u) were 90 ± 2 % with 2 μM acetylcholine and 16 ± 3 % with 200 μM acetylcholine.

		α4β2			α4(u)β2(u)		
[acetylcholine]	**antagonist**	**IC_{50}, μM**	**nH**	**N**	**IC_{50}, μM**	**nH**	**N**
2 μM	DHβE	0.0023 (0.0010-0.0050)	0.83 ± 0.20	3	0.0027 (0.0020-0.0036)	1.20 ± 0.18	3
	MLA	0.063 (0.049-0.081)	1.29 ± 0.18	3	0.13 (0.11-0.16)	1.52 ± 0.18	3
	Mec	0.58 (0.27-1.26)	0.65 ± 0.14	3	2.0 (1.2-3.3)	0.72 ± 0.12	3
	dTC	110 (11-1200)	0.41 ± 0.30	3	4.7 (3.2-7.0)	0.78 ± 0.11	4
	CI	0.52 (0.34-0.80)	0.63 ± 0.08	5	18 (13-26)	1.00 ± 0.16	5

		α4β2			α4(u)β2(u)		
[acetylcholine]	**antagonist**	**IC_{50}, μM**	**nH**	**N**	**IC_{50}, μM**	**nH**	**N**
200 μM	DHβE	0.0056 (0.0021-0.015)	0.60 ± 0.15	3	0.0036 (0.0020-0.0062)	0.95 ± 0.21	3
	MLA	0.038 (0.032-0.045)	1.23 ± 0.11	3	0.101 (0.073-0.139)	1.23 ± 0.21	3
	Mec	0.24 (0.21-0.28)	0.90 ± 0.05	6	3.81 (2.67-5.64)	1.10 ± 0.21	6
	dTC	0.92 (0.69-1.23)	0.75 ± 0.07	5	49.7 (8.8-280)	0.39 ± 0.13	3
	CI	0.18 (0.17-0.20)	1.14 ± 0.05	6	3.3 (2.9-3.7)	0.84 ± 0.03	2

Figure 1



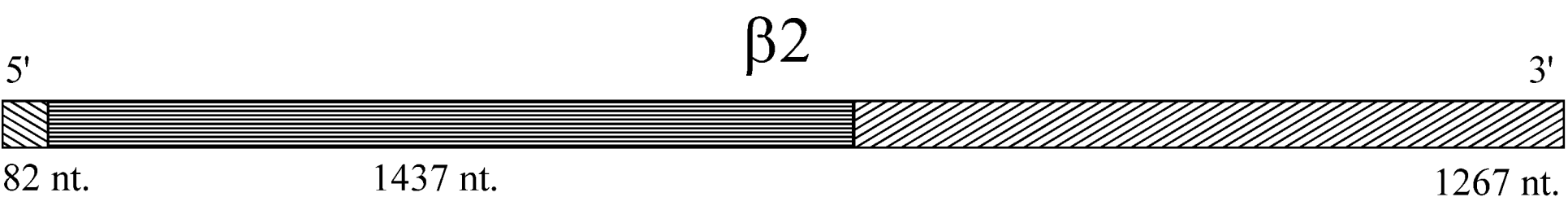
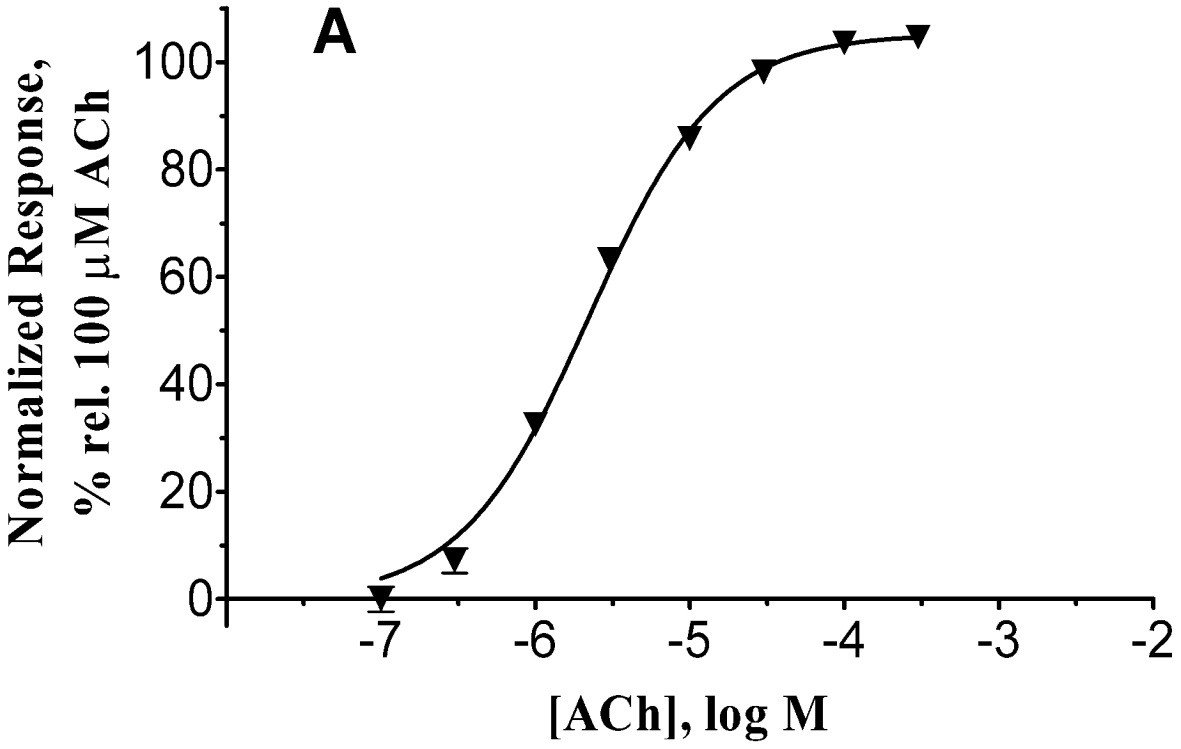


Figure 2


A
Normalized Response,
% rel. 100 μM ACh
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
[ACh], log M

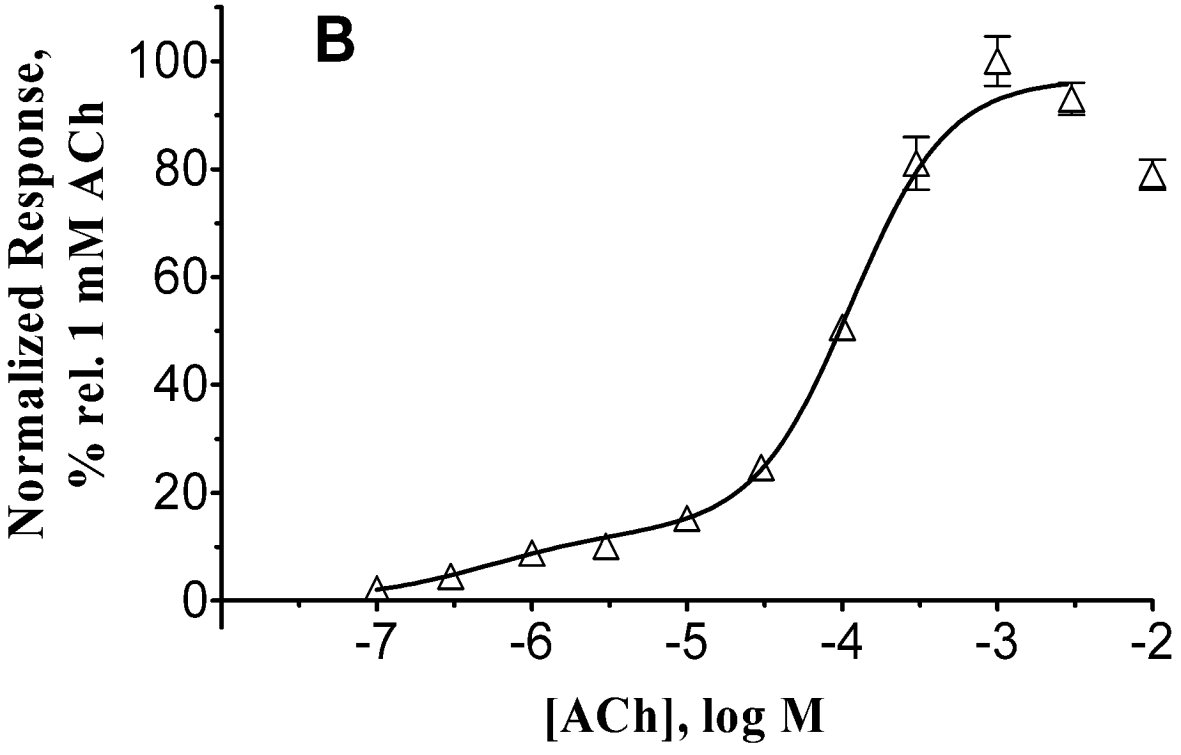
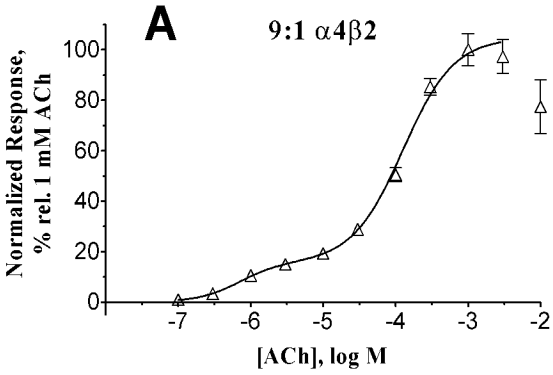
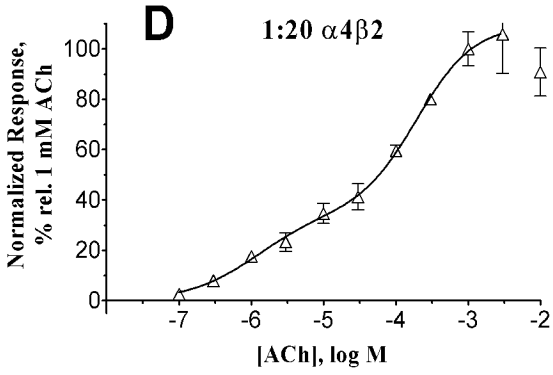
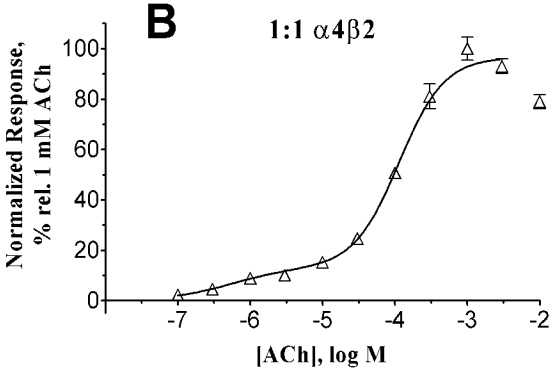
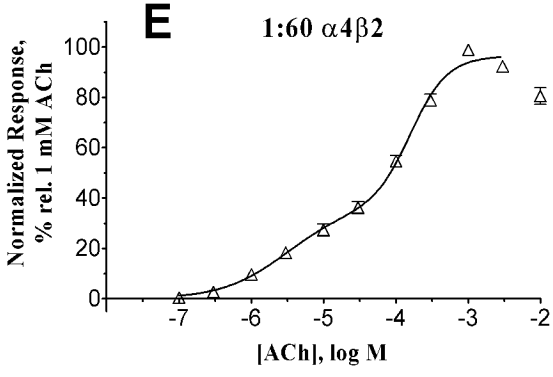

B
Normalized Response,
% rel. 1 mM ACh
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
[ACh], log M

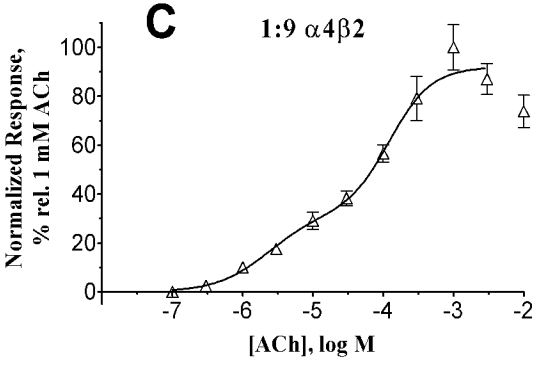
Figure 3


A
9:1 α4β2
Normalized Response, % rel. 1 mM ACh
[ACh], log M


D
1:20 α4β2
Normalized Response, % rel. 1 mM ACh
[ACh], log M


B
1:1 α4β2
Normalized Response, % rel. 1 mM ACh
[ACh], log M


E
1:60 α4β2
Normalized Response, % rel. 1 mM ACh
[ACh], log M


C
1:9 α4β2
Normalized Response, % rel. 1 mM ACh
[ACh], log M

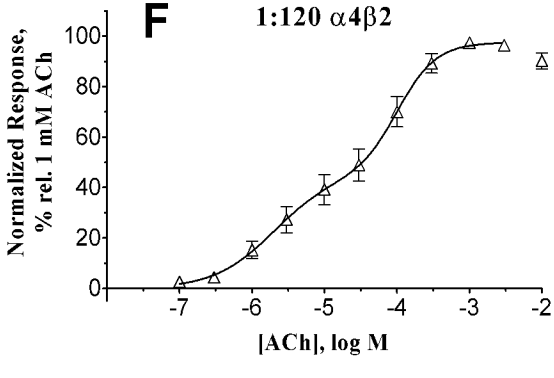
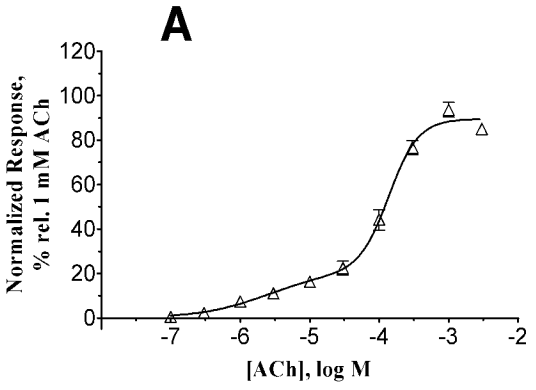
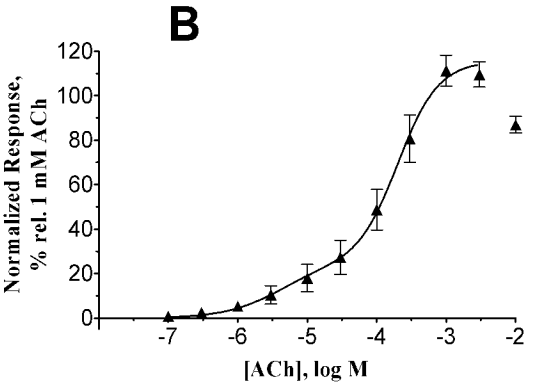
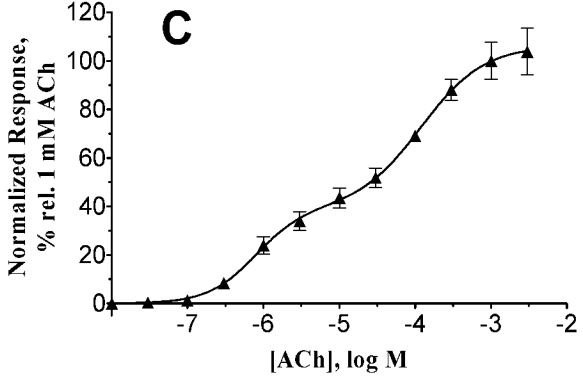

F
1:120 α4β2
Normalized Response, % rel. 1 mM ACh
[ACh], log M

Figure 4


A
Normalized Response, % rel. 1 mM ACh
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
[ACh], log M


B
Normalized Response, % rel. 1 mM ACh
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
[ACh], log M


C
Normalized Response, % rel. 1 mM ACh
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
[ACh], log M

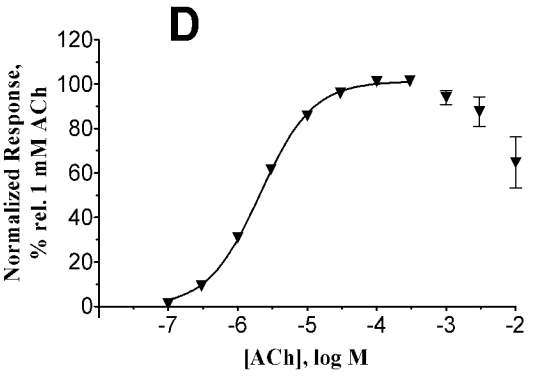

D
Normalized Response, % rel. 1 mM ACh
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
[ACh], log M

Figure 5

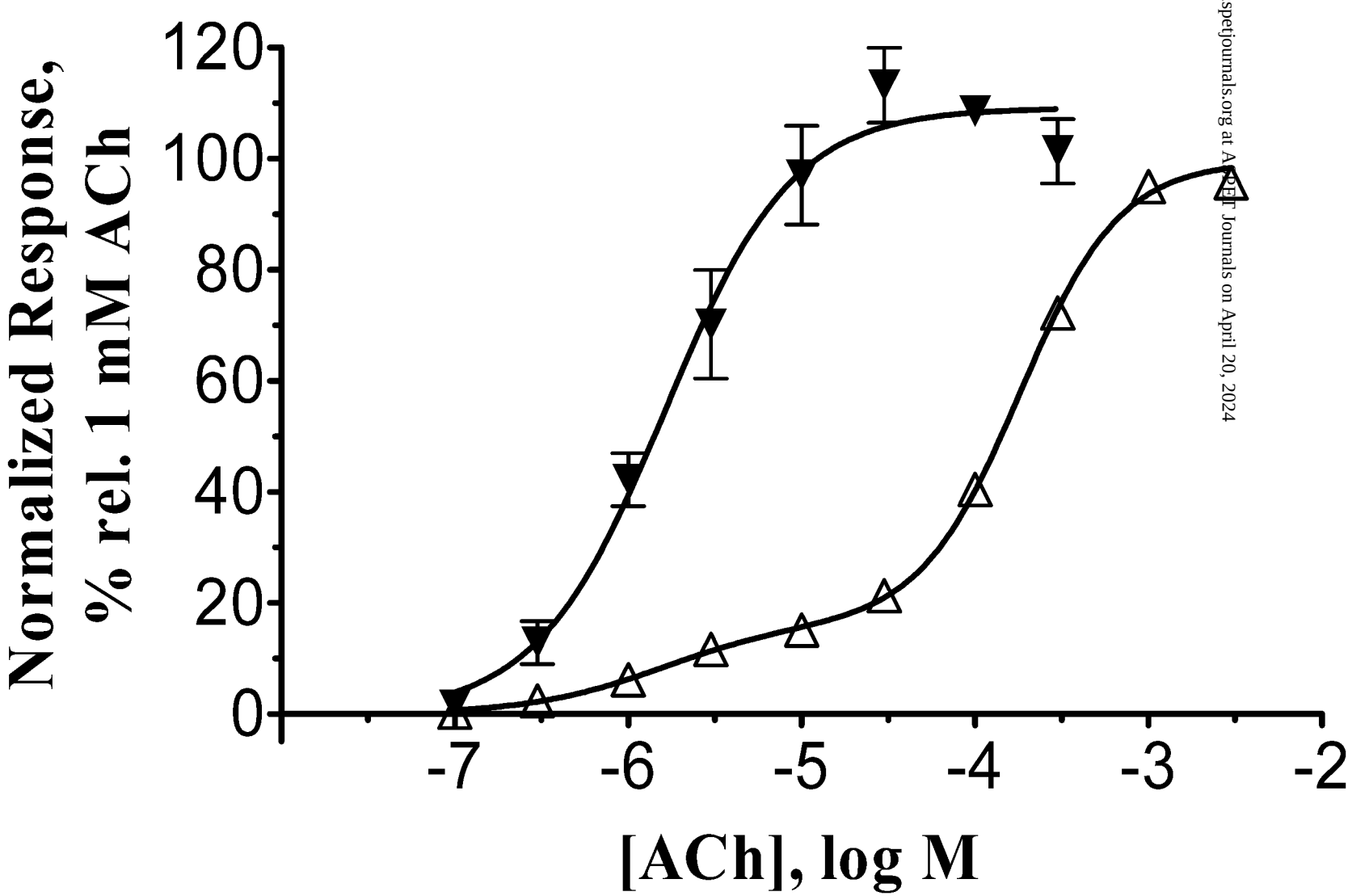
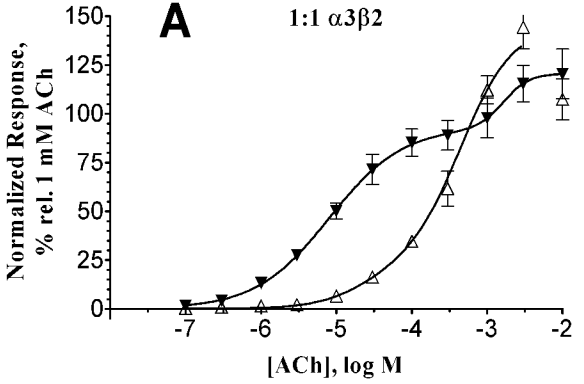
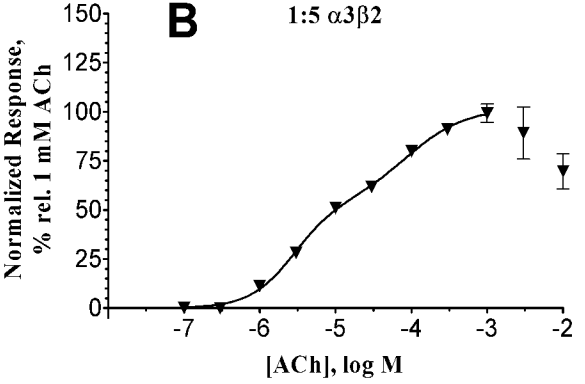
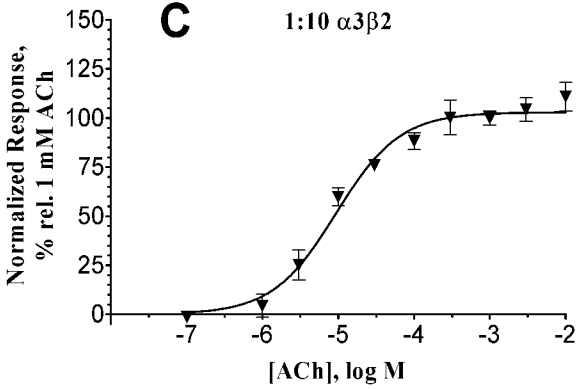


Figure 6


A
1:1 α3β2
Normalized Response, % rel. 1 mM ACh
150
125
100
75
50
25
0
-7
-6
-5
-4
-3
-2
[ACh], log M


B
1:5 α3β2
Normalized Response, % rel. 1 mM ACh
150
125
100
75
50
25
0
-7
-6
-5
-4
-3
-2
[ACh], log M


C
1:10 α3β2
Normalized Response, % rel. 1 mM ACh
150
125
100
75
50
25
0
-7
-6
-5
-4
-3
-2
[ACh], log M

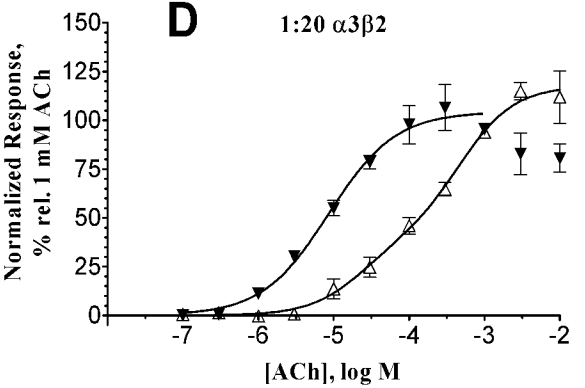
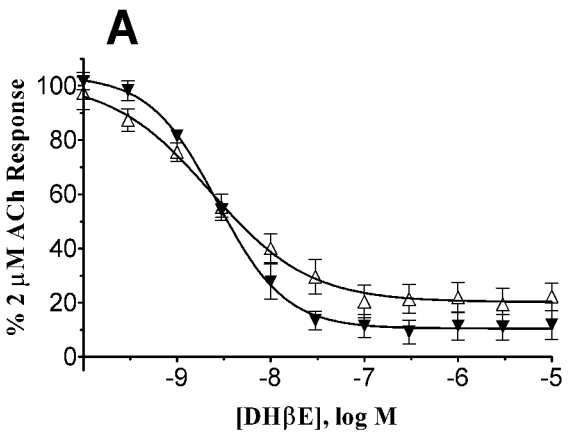
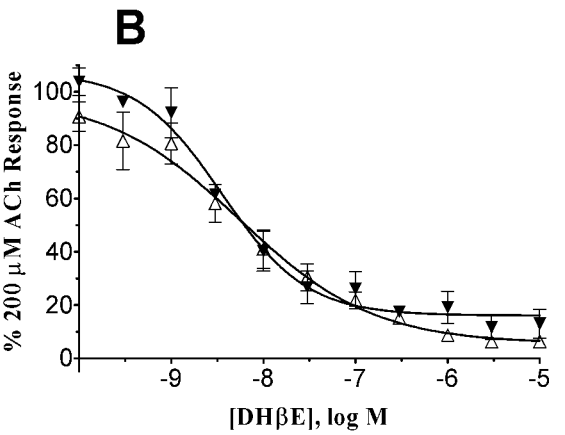
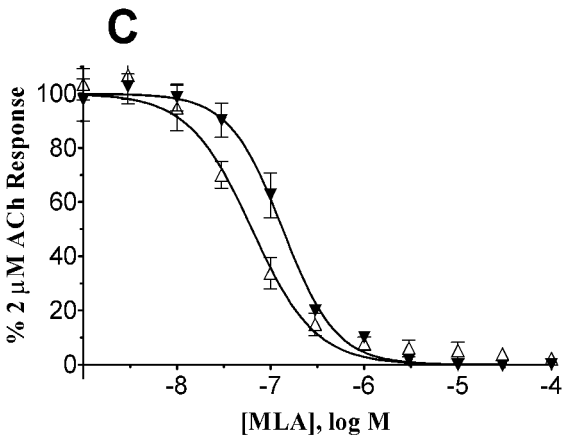

D
1:20 α3β2
Normalized Response, % rel. 1 mM ACh
150
125
100
75
50
25
0
-7
-6
-5
-4
-3
-2
[ACh], log M

Figure 7


A
% 2 μM ACh Response
100
80
60
40
20
0
-9
-8
-7
-6
-5
[DHβE], log M


B
% 200 μM ACh Response
100
80
60
40
20
0
-9
-8
-7
-6
-5
[DHβE], log M


C
% 2 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[MLA], log M

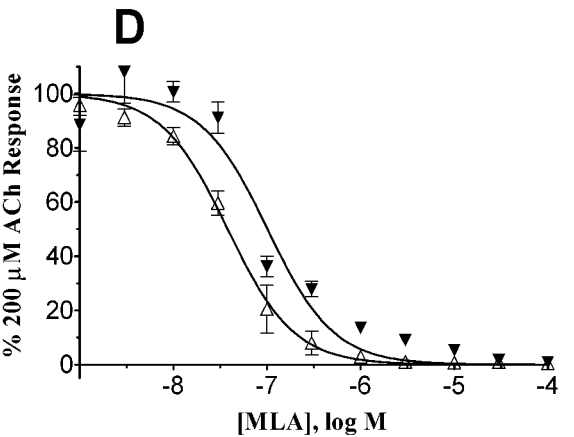
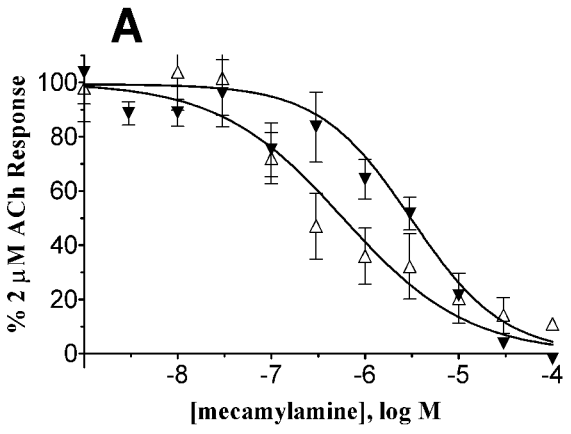
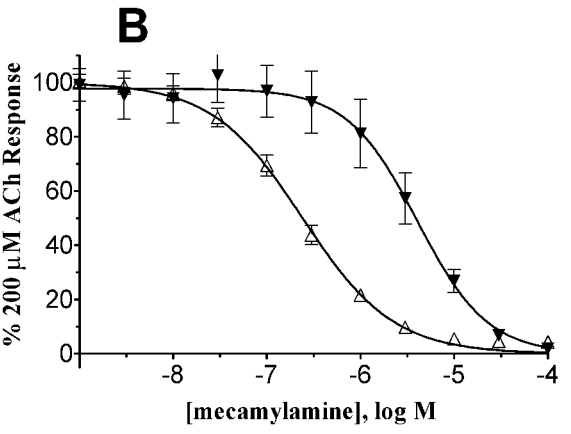
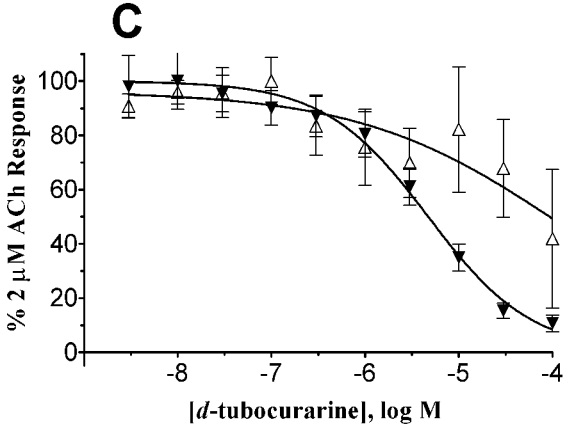
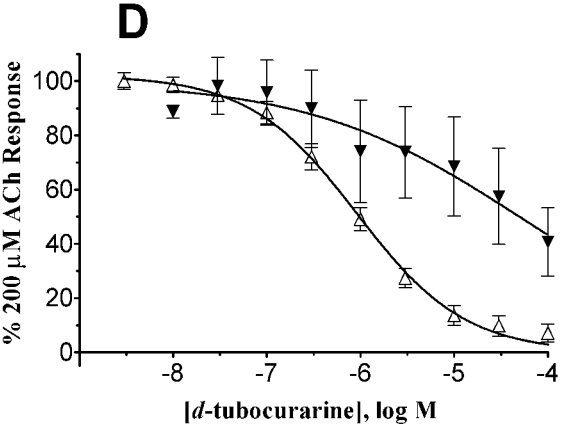

D
% 200 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[MLA], log M

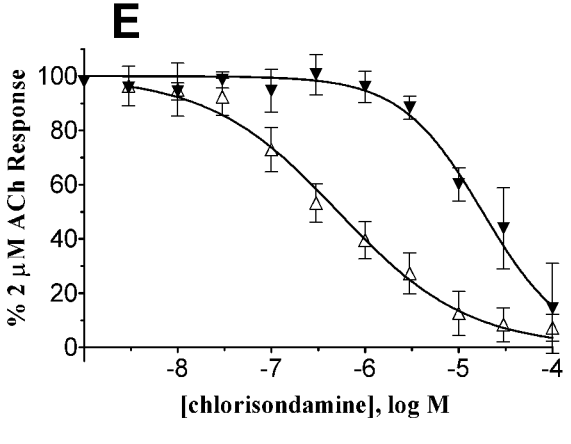
Figure 8


A
% 2 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[mecamylamine], log M


B
% 200 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[mecamylamine], log M


C
% 2 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[d-tubocurarine], log M


D
% 200 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[d-tubocurarine], log M


E
% 2 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[chlorisondamine], log M

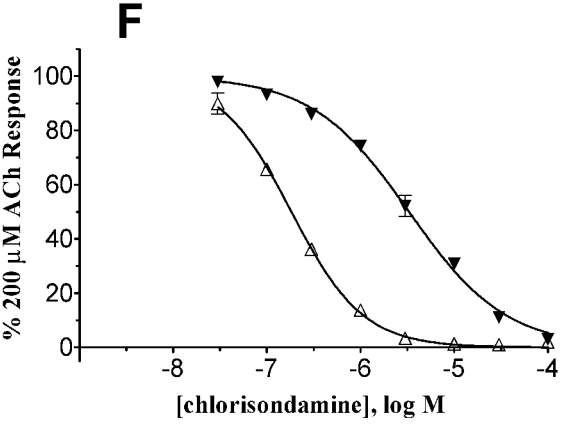
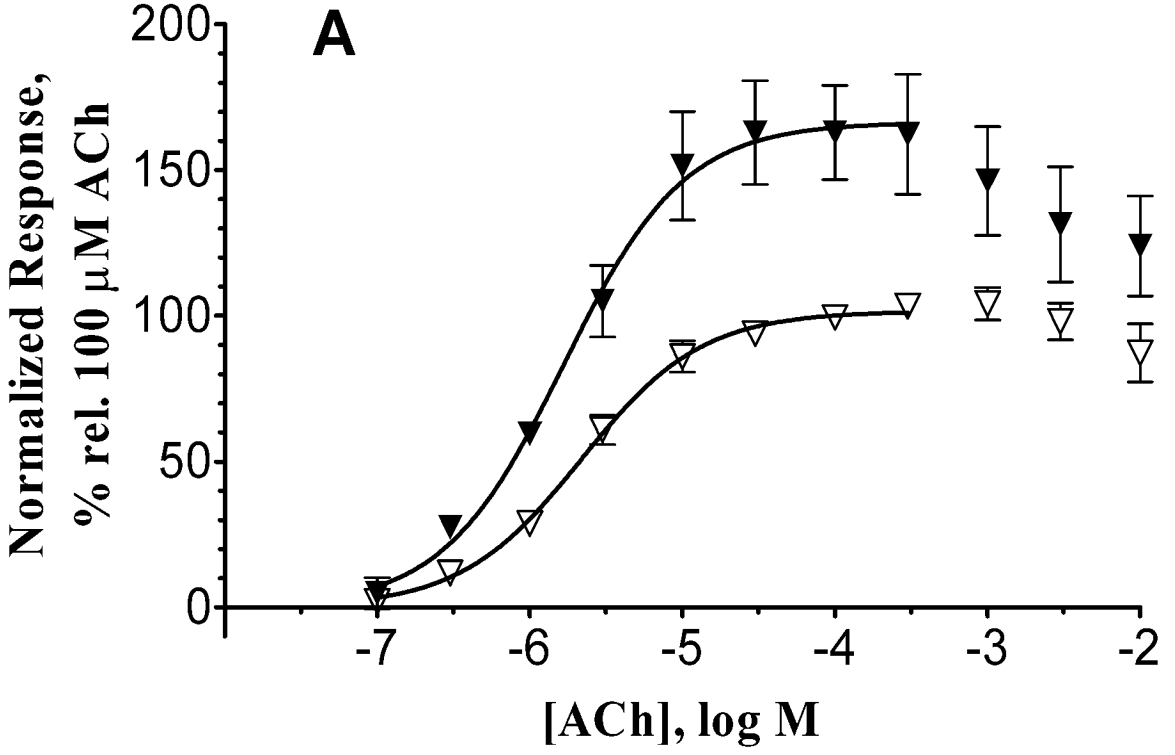

F
% 200 μM ACh Response
100
80
60
40
20
0
-8
-7
-6
-5
-4
[chlorisondamine], log M

Figure 9


A
Normalized Response,
% rel. 100 μM ACh
200
150
100
50
0
-7
-6
-5
-4
-3
-2
[ACh], log M

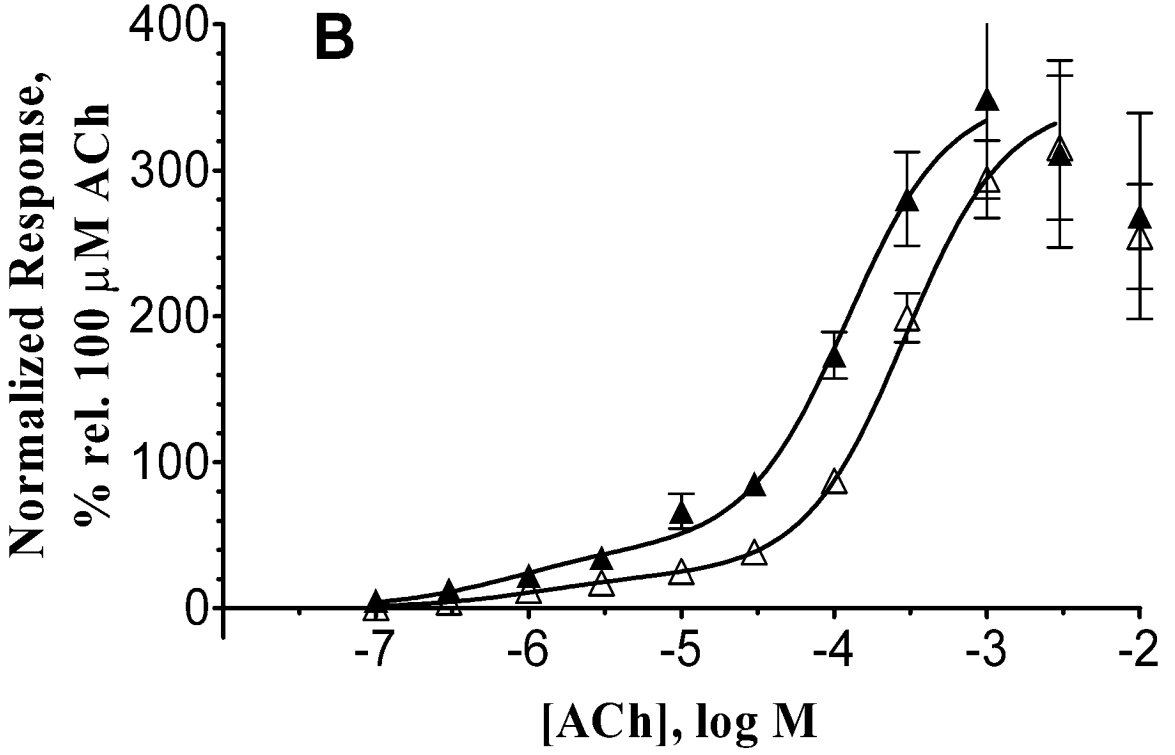

B
Normalized Response,
% rel. 100 μM ACh
400
300
200
100
0
-7
-6
-5
-4
-3
-2
[ACh], log M

Figure 10

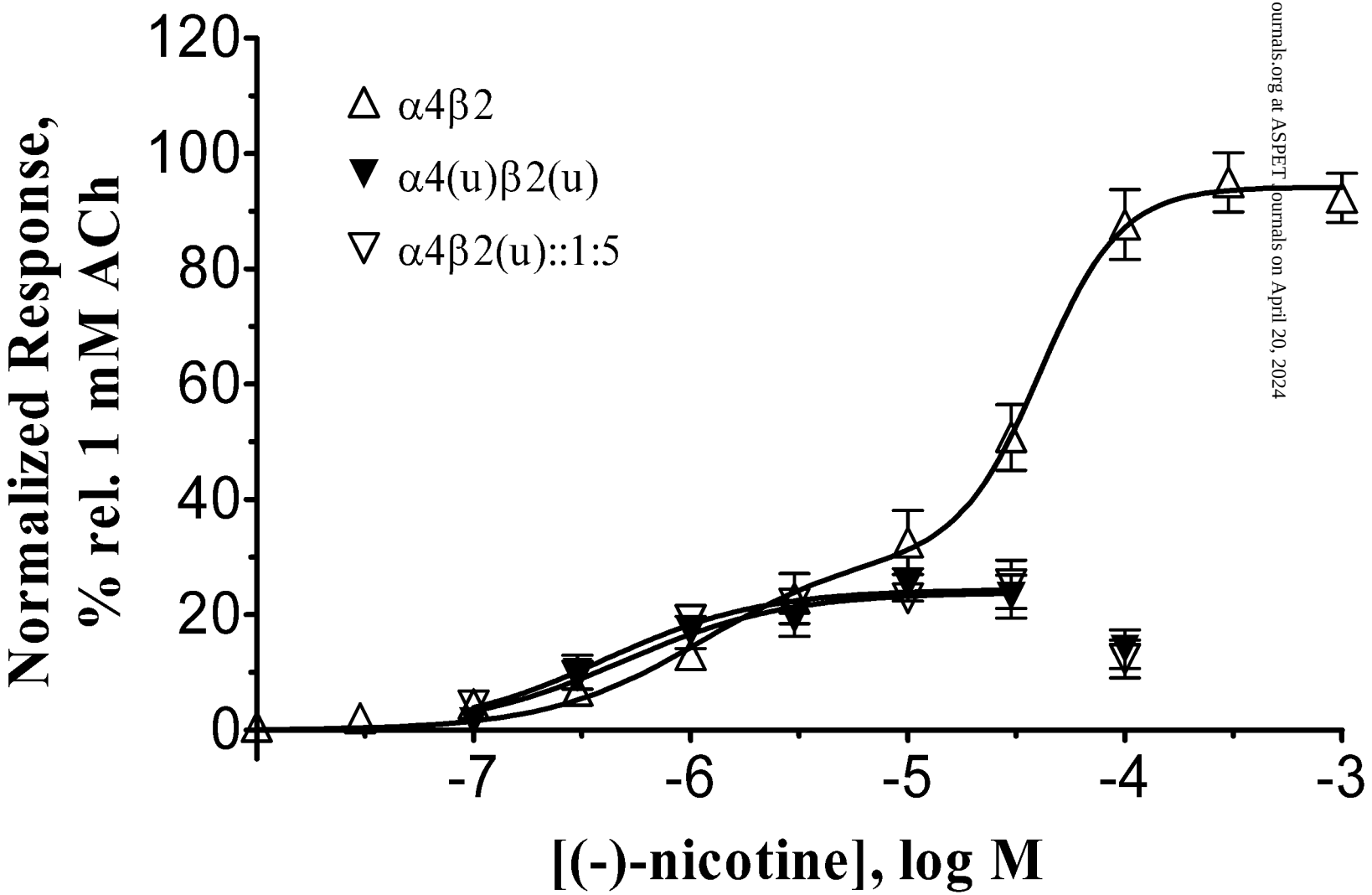


Figure 11

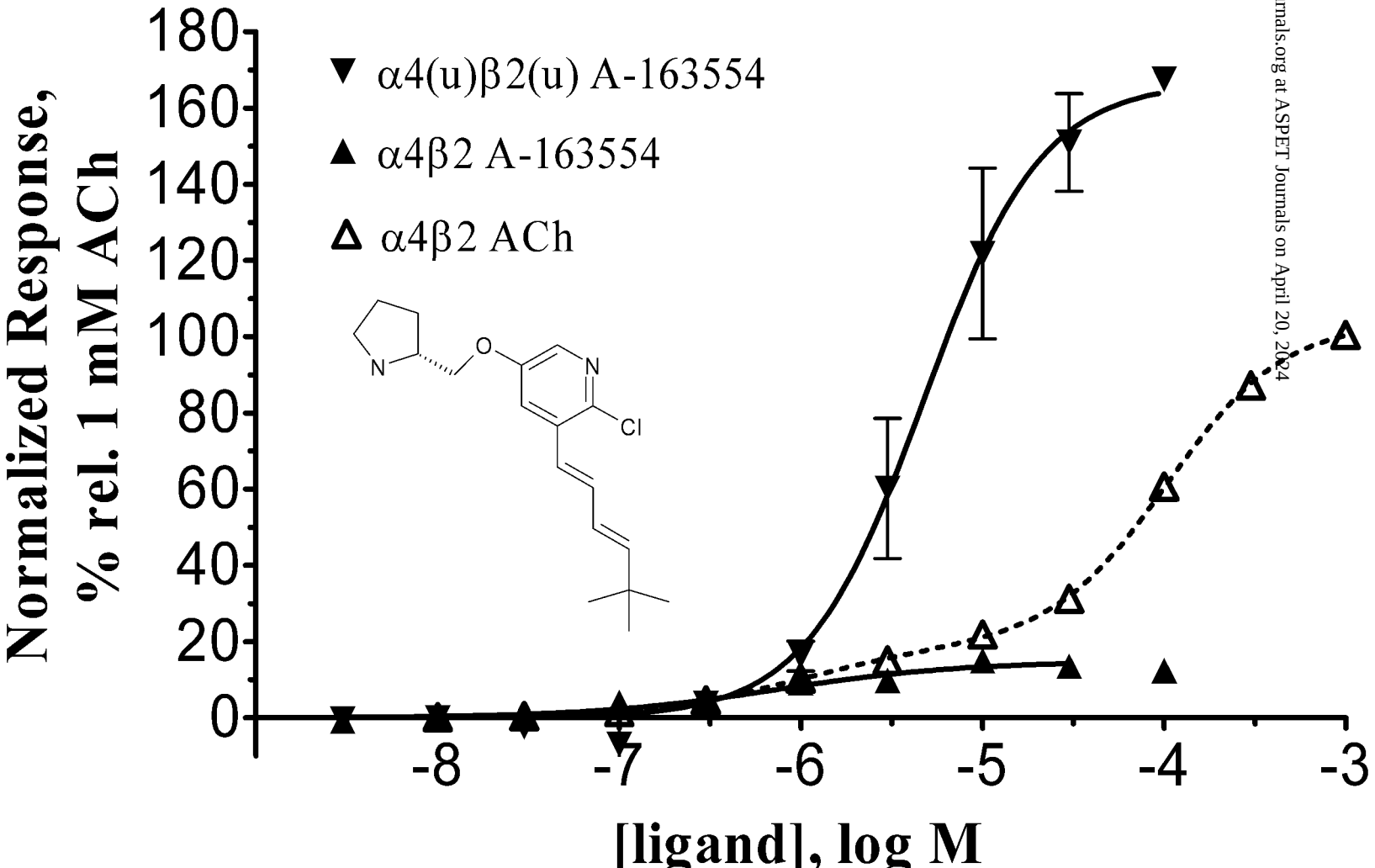


Figure 12

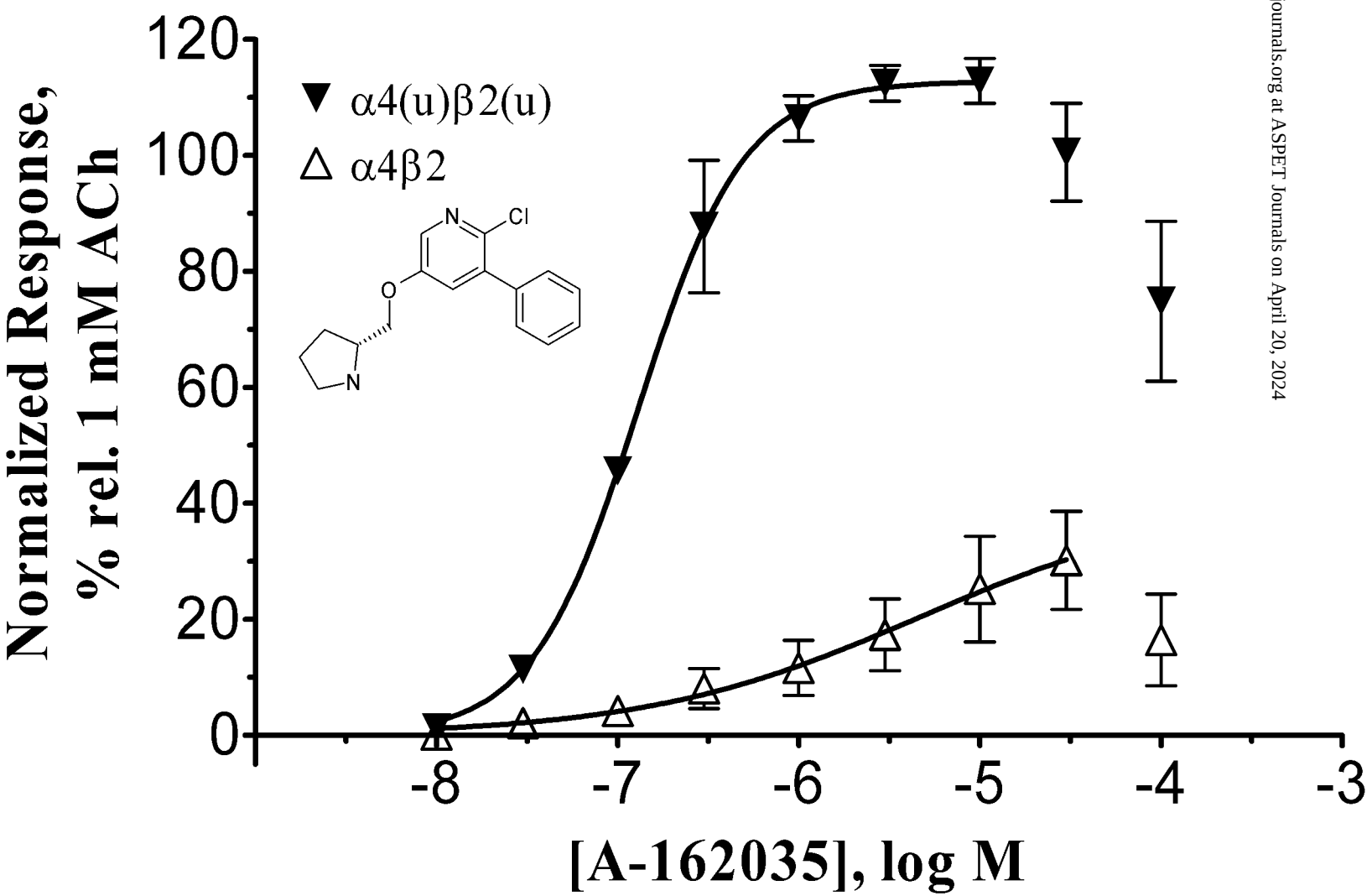


Figure 13

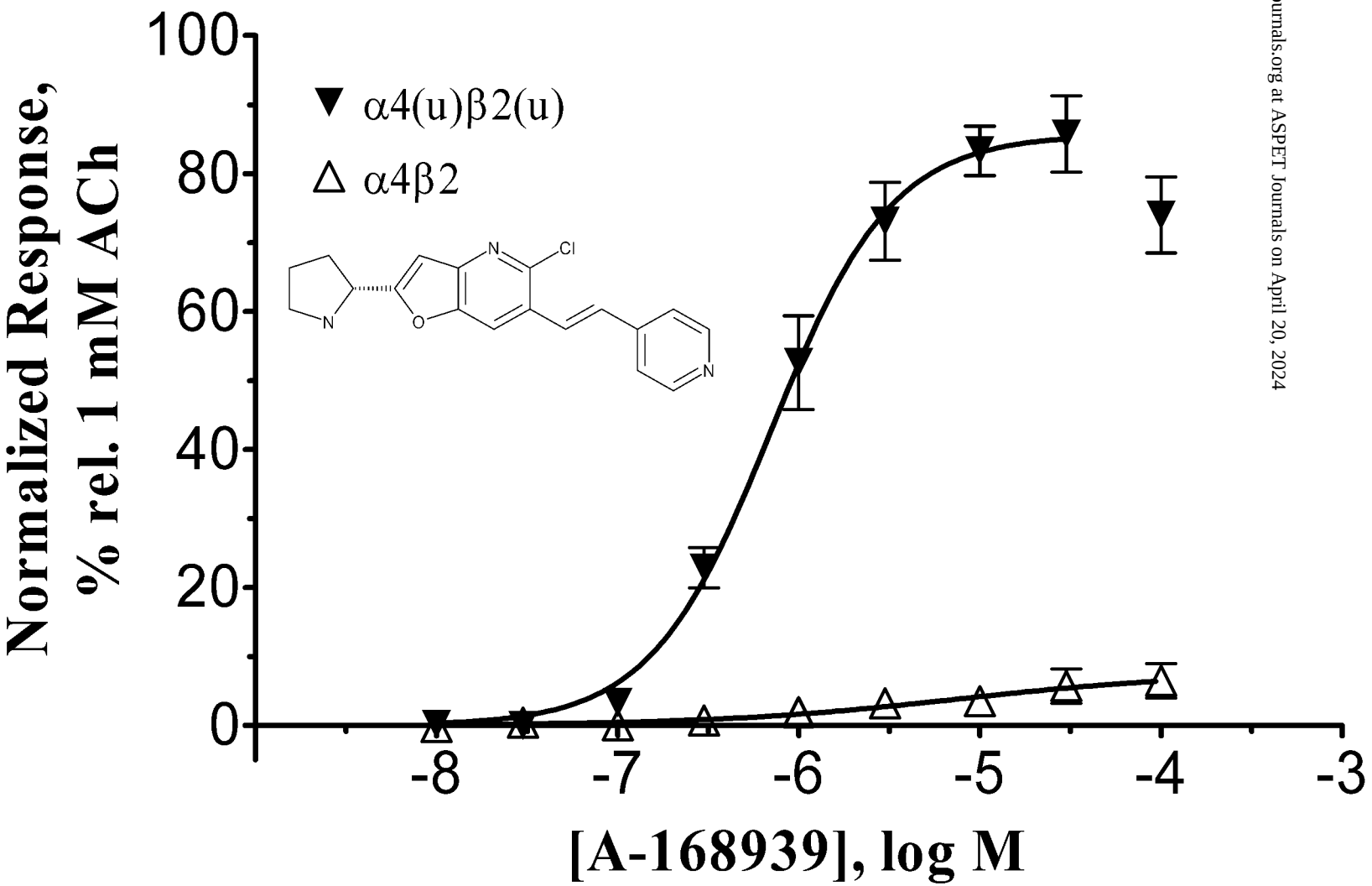
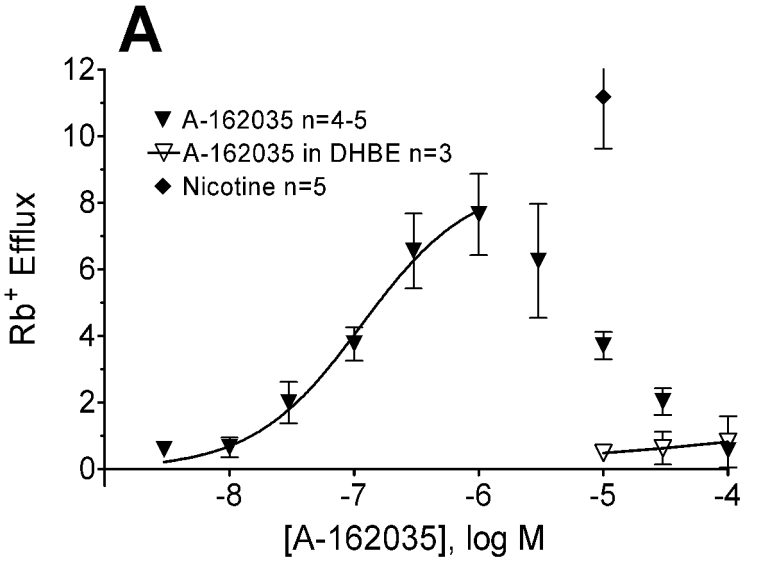
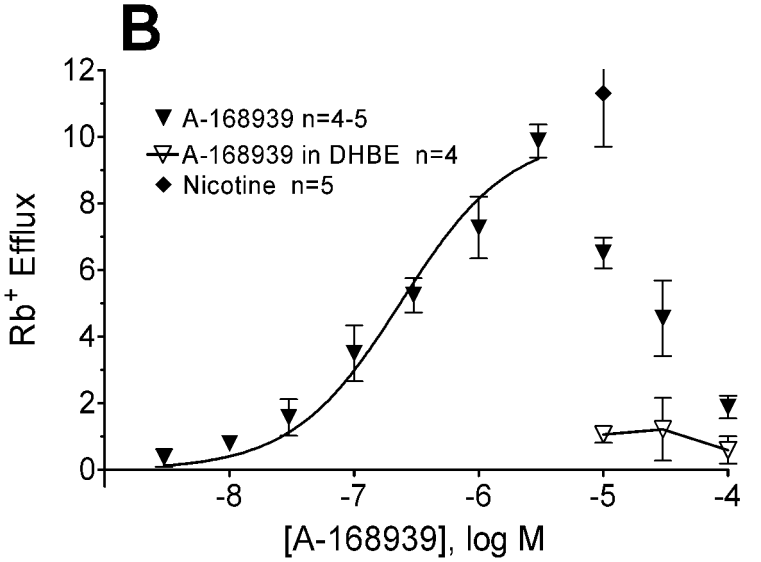
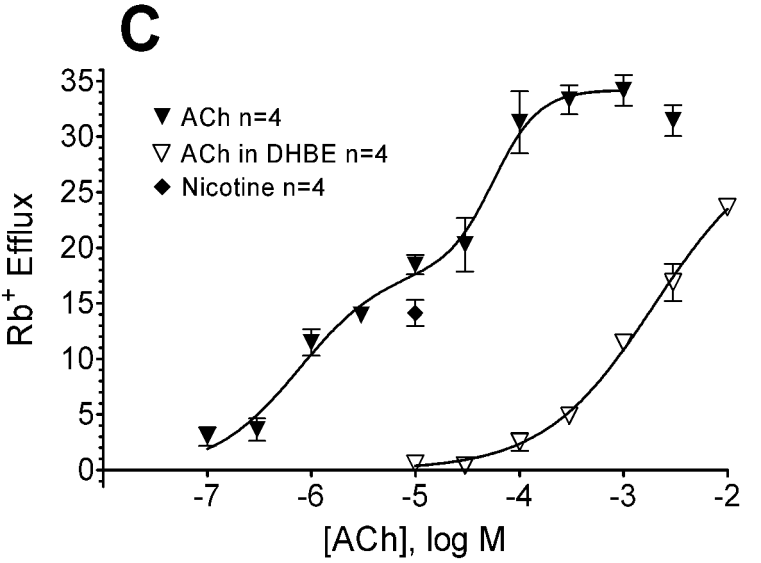


Figure 14


A
A-162035 n=4-5
A-162035 in DHBE n=3
Nicotine n=5
Rb+ Efflux
[A-162035], log M


B
A-168939 n=4-5
A-168939 in DHBE n=4
Nicotine n=5
Rb+ Efflux
[A-168939], log M


C
ACh n=4
ACh in DHBE n=4
Nicotine n=4
Rb+ Efflux
[ACh], log M