

**Untranslated Region Dependent Exclusive Expression of High-Sensitivity Subforms of  
 $\alpha 4\beta 2$  and  $\alpha 3\beta 2$  Nicotinic Acetylcholine Receptors**

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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- $\beta$ -erythroidine (DH $\beta$ E); mecamylamine (Mec); methyllycaconitine (MLA); nicotinic acetylcholine receptor (nAChR); open reading frame (ORF); *d*-tubocurarine (dTC); untranslated region (UTR);

### Abstract

$\alpha 4\beta 2$  nicotinic acetylcholine receptors are recognized as the principal nicotine binding site in brain. Recombinant  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  and  $\alpha 3\beta 2$  nAChR. Oocytes injected with  $\alpha 4$  and  $\beta 2$  RNA lacking UTR expressed biphasic concentration-response relationships for acetylcholine with high-sensitivity  $EC_{50}$  values of 0.5 to 2.5  $\mu M$  (14-24% of the population) and low-sensitivity  $EC_{50}$  values of 110-180  $\mu M$  (76-86%). In contrast, message with UTR expressed exclusively the high-sensitivity  $\alpha 4\beta 2$  nAChR subform with an acetylcholine  $EC_{50}$  value of 2.2  $\mu M$ . Additional studies revealed pharmacologic differences between high- and low-sensitivity  $\alpha 4\beta 2$  subforms. While the antagonists dihydro- $\beta$ -erythroidine ( $IC_{50}$  3-6 nM) and methyllycaconitine ( $IC_{50}$  40-135 nM) were not selective between high- and low-sensitivity  $\alpha 4\beta 2$ , chlorisondamine, mecamylamine and *d*-tubocurarine were, respectively, 100-, 8-, and 5-fold selective for the  $\alpha 4\beta 2$  subform with low-sensitivity to acetylcholine. Conversely, agonists that selectively activated the high-sensitivity  $\alpha 4\beta 2$  subform with respect to efficacy as well as potency were identified. Further, two of these agonists were shown to activate mouse brain  $\alpha 4\beta 2$  as well as the ferret high-sensitivity  $\alpha 4\beta 2$  expressed in *Xenopus* oocytes. Using UTR-containing RNA, exclusive expression of a novel high-sensitivity  $\alpha 3\beta 2$  nAChR was also achieved. These studies (a) provide further evidence for the existence of multiple subforms of  $\alpha 4\beta 2$  nAChR, (b) extend that to  $\alpha 3\beta 2$  nAChR, (c) demonstrate UTR influence on  $\beta 2$ -containing

nAChR properties, and (d) reveal compounds that interact with  $\alpha 4\beta 2$  in a subform-selective manner.



## 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<sub>3</sub>, GABA<sub>A</sub> and glycine receptors. Functional receptors are comprised by at least one “ $\alpha$ ” subunit which contains signature sequences required for binding and channel activation. However, most nAChR also require non- $\alpha$  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.  $\alpha 4_{(2)}\beta 2_{(3)}$  and  $\alpha 4_{(3)}\beta 2_{(2)}$  (Zhou et al., 2003). In mammalian brain, nine subunits predominate -  $\alpha 2$  through  $\alpha 7$  and  $\beta 2$  through  $\beta 4$ , and among these only  $\alpha 7$  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  $\alpha 4\beta 2$ ,  $\alpha 3$ -containing ( $\alpha 3^*$ ),  $\alpha 6^*$ , and  $\alpha 7$  nAChR. In particular, about 90% of the high-affinity nicotine binding sites in rat brain comprise  $\alpha 4\beta 2$  (Champtiaux et al., 2003; Zoli et al., 1995; Flores et al., 1992; Whiting et al., 1987; Clarke et al., 1985). Functionally, native  $\alpha 4\beta 2$  nAChR EC<sub>50</sub> 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  $\alpha 4\beta 2$ . In contrast, recombinant  $\alpha 4\beta 2$  expressed in oocytes and mammalian cell lines have demonstrated variable

functional potencies for acetylcholine and nicotine with lower sensitivity  $EC_{50}$  values  $> 40 \mu\text{M}$  (Papke et al., 2000; Sabey et al., 1999; Chavez-Noriega et al., 1997; Gopalakrishnan et al., 1996) as well as the higher sensitivity  $\leq 3 \mu\text{M}$   $EC_{50}$  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  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$  (Houlihan et al., 2001; Covernton and Connolly, 2000) in a proportion that may be influenced by  $\alpha 4$  polymorphism (Kim et al., 2003), by  $\beta 2$  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  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  nAChR subform. This property does not appear to be limited to  $\alpha 4\beta 2$  nAChR, but extends at least to  $\alpha 3\beta 2$  nAChR as well.

Among the various nAChR,  $\alpha 4\beta 2$  are unusual in that they are potentiated rather than inhibited by the neuroactive steroid  $17\beta$ -estradiol (Curtis et al., 2002; Nakazawa and Ohno, 2001) through a mechanism involving the carboxy-terminus of the  $\alpha 4$  subunit (Paradiso et al., 2001). Estradiol also potentiated ferret  $\alpha 4\beta 2$  nAChR, and with apparently greater effect on the high-sensitivity

subform. Thus,  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  nAChR. With both high-sensitivity and mixed-sensitivity forms of  $\alpha 4\beta 2$ , dihydro- $\beta$ -erythroidine (DH $\beta$ E) 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  $\alpha 4\beta 2$ , 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  $\alpha 4\beta 2$  nAChR subform. Two of these agonists were shown to be active at mouse brain  $\alpha 4\beta 2$  nAChR as well as at ferret high-sensitivity  $\alpha 4\beta 2$  nAChR expressed in oocytes, supporting the idea that the high-sensitivity  $\alpha 4\beta 2$  nAChR subform is expressed in brain.

## **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  $\alpha 4$  and  $\beta 2$  provided coding sequence and genomic 5'- and 3'-untranslated regions (UTR) while the PCR methodology utilized for  $\alpha 3$ ,  $\alpha 4$  and  $\beta 2$  generated coding sequence without the UTR.

### **Isolation of $\alpha 4$ and $\beta 2$ 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

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  $\gamma$ -<sup>32</sup>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  $\alpha$  and  $\beta$  nAChR subunits)

Oligonucleotide 2: GAACGGTTGCTGAAGACACTCTTCTCCGGCTACAACAAGTGGTC

(ferret  $\alpha$ 4, N terminal half)

Oligonucleotide 3: GGCGGCTCATCGAGTCCATGCACAAGGTGGCCAGCGCCCC

(ferret  $\alpha 4$ , C terminal half)

Oligonucleotide 4: GAGCGGCTAGTGGAGCATCTCCTGGACCCCTCCCGGTACAACAAG

(ferret  $\beta 2$ , N terminal half)

Oligonucleotide 5: ACCATCGGCATGTTTCCTGCAGCCTCTCTTCCAGAACTACAC

(human  $\beta 2$ , C terminal half).

Based upon hybridization signals, individual library aliquots believed to contain full-length  $\alpha 4$  and  $\beta 2$  subunit cDNA clones were identified. Colonies from each were plated onto agar, grown, transferred to nylon membranes and screened with oligonucleotide probes. For  $\alpha 4$  a mixture of oligonucleotides 1, 2 and 3 was used; for  $\beta 2$  a mixture of oligonucleotides 1, 4 and 5 was used. Individual colonies were identified and characterized. All  $\alpha 4$  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  $\beta 2$ ; 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  $\beta 2$  clone.

## **Isolation of $\alpha 3$ , $\alpha 4$ and $\beta 2$ cDNAs by PCR**

cDNA prepared from either total RNA or poly-A<sup>+</sup> 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  $\beta 2$ , 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  $\alpha 4$  and  $\alpha 3$ , 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  $\mu$ l 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  $\alpha 3$  nAChR subunit, four overlapping partial cDNA clones were used to construct a full-length clone, for the  $\alpha 4$  subunit three overlapping clones were used, and for the  $\beta 2$  cDNA four overlapping clones were used. Primer sequences used to generate these partial cDNAs were as follows.

$\alpha 3$  nAChR primers

- Set 1: 5'-CTCCAGGTCTGGGGTCTGCGCTG-3' (sense),  
5'-GCTTTGGTCTTGTCGTCCACCTGG-3' (antisense)
- Set 2: 5'-GCCAGTGGCCAGGGCCTCAGAGGC-3' (sense),  
5'-CCCAGTAGTCCTTGAGGTTTCATGG-3' (antisense)
- Set 3: 5'-CCATGAACCTCAAGGACT-3' (sense)  
5'-CACCATGGCAACATATTCC-3' (antisense)
- Set 4: 5'-GCCAAAGAGATTCAAGATGATTGGAAGTATGTTGCCATGG-3' (sense)  
5'-TCTATGTGTCATCTCTGGCCATCAAGGGTTGCAG-3' (antisense)

$\alpha 4$  nAChR primers

- Set 1: 5'-TGCGTGCGCCATGGAGCTAGGGGGC-3' (sense)  
5'-CGTACGTCCAGGAGCCGAACCTTCATG-3' (antisense)
- Set 2: 5'-ACGGRMGGGTGCAGTGGA-3' (sense)  
5'-CTTCTGGCCMGAGCCWG-3' (antisense)



Set 3: 5'-CGGCCCTCCGTGGTCAAGGACAAC-3' (sense)

5'-TCCTAGATCATRCCAGCCA-3' (antisense)

### $\beta$ 2 nAChR primers

Set 1: 5'-CGGCTTCAGCACCCACGGACAGCGCCCCACC-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  $\alpha$ 4 and  $\alpha$ 3 subunits) or pcDNA3.1(-) (for the  $\beta$ 2 subunit). Capped cRNA was prepared using mMessage mMachine (Ambion, Austin, TX) transcription via the T7 promoter in the pcDNA 3.1 vector.

### **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<sup>2+</sup> Barth's solution (pH 7.55) containing 87.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 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<sub>3</sub>, 0.74 mM CaCl<sub>2</sub>, 0.82 mM MgCl<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>, 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

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<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 5 mM Na-HEPES buffer, and 0.5 μM atropine to block endogenous muscarinic receptors. In some experiments, CaCl<sub>2</sub> was replaced by BaCl<sub>2</sub> in order to prevent secondary activation of a Ca<sup>2+</sup>-dependent Cl<sup>-</sup> 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.

In each oocyte, responses to test compound were normalized to the maximal response to acetylcholine (100  $\mu$ M 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  $\alpha 4\beta 2$  nAChR, DH $\beta$ E-sensitive stimulation of  $^{86}\text{Rb}^+$  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  $^{86}\text{Rb}^+$ , the P2 was resuspended in uptake buffer (140 mM NaCl, 1.5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1

mM MgSO<sub>4</sub>, 20 mM glucose and 25 mM Na-HEPES buffer, pH 7.5) and incubated with 4 μCi <sup>86</sup>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<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 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 <sup>86</sup>Rb<sup>+</sup> 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

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  $^{86}\text{RbCl}$  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).

## Results

In cloning ferret  $\alpha 4$  and  $\beta 2$  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,  $\alpha 4$  and  $\beta 2$  messages with relatively long 3' and 5' UTR were isolated by the cDNA library screening. The relative sizes of the ferret  $\alpha 4$  and  $\beta 2$  nAChR UTR segments are diagrammed in **Figure 1**. The **Supplemental Information** shows ferret  $\alpha 3$ ,  $\alpha 4$  and  $\beta 2$  nAChR amino acid sequences and ferret  $\alpha 4$  and  $\beta 2$  nAChR UTR nucleotide sequences aligned with corresponding human and rat sequences.

### **Expression of high- and low-sensitivity $\alpha 4\beta 2$ nAChR**

Oocytes injected with RNA or DNA derived from these clones expressed functional  $\alpha 4\beta 2$  nAChRs, but with different results depending upon whether or not the messages contained UTR sequences. In the following, " $\alpha 4(u)$ " refers to  $\alpha 4$  coding sequence with 5' and 3' UTR; likewise, " $\beta 2(u)$ " refers to  $\beta 2$  coding sequence with 5' and 3' UTR.

Oocytes injected with ferret  $\alpha 4$  and  $\beta 2$  (1:1 ratio) lacking UTR expressed typical acetylcholine-gated currents and a biphasic concentration-response relationship as reported previously using human and rat  $\alpha 4\beta 2$  (Nelson et al., 2003; Buisson and Bertrand, 2001; Chavez-Noriega et al.,

2000; Zwart and Vijverberg, 1998). In contrast, when  $\alpha 4(u)$  and  $\beta 2(u)$  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  $\alpha 4(u)\beta 2(u)$  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  $\beta 2$  message to an  $\alpha 4:\beta 2$  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  $\beta 2$  protein due to increased translation of  $\beta 2$  due to the presence of UTR, we attempted to generate monophasic high-sensitivity acetylcholine concentration curves by adjusting the  $\alpha 4:\beta 2$  ratio using messages without UTR. Decreasing the  $\alpha 4:\beta 2$  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  $\alpha 4\beta 2$  using messages lacking UTR.

To determine whether  $\alpha 4$  UTR or  $\beta 2$  UTR was required for exclusive expression of the high-sensitivity  $\alpha 4\beta 2$  subform,  $\alpha 4$  with or without UTR was combined with  $\beta 2$  with or without UTR. Using 1:1 ratios, UTR in both  $\alpha 4$  and  $\beta 2$  appeared to be required (**Figure 4**) because the low-sensitivity subform clearly was expressed when either  $\alpha 4$  without UTR or  $\beta 2$  without UTR was used. However, the  $\beta 2$  with UTR appeared to have the greater effect and could increase the



expression of the high-sensitivity  $\alpha 4\beta 2$  subform even when  $\alpha 4$  lacked UTR (**Figure 4C** and **Table 1**). Consistent with this, in further experiments it was found that high-sensitivity  $\alpha 4\beta 2$  could be exclusively expressed using  $\alpha 4$  lacking UTR plus  $\beta 2$  with UTR in a ratio of 1:5:: $\alpha 4$ : $\beta 2(u)$  (**Figure 5**).

### **Expression of high- and low-sensitivity $\alpha 3\beta 2$ nAChR**

The above observations suggested that  $\beta 2(u)$  could regulate the form of  $\alpha 4\beta 2$  nAChR expressed in oocytes. To determine whether this effect may generalize to other  $\beta 2$ -containing nAChR, ferret  $\alpha 3$  was combined with  $\beta 2$  and  $\beta 2(u)$  in ratios ranging from 1:1 to 1:20:: $\alpha 3$ : $\beta 2$ . The acetylcholine-concentration-response curve for  $\alpha 3\beta 2$  1:1 could be fit with a biphasic curve and  $EC_{50}$ s of 25 and 450  $\mu M$  (**Figure 6 and Table 2**). Using  $\beta 2(u)$ , however, a lower  $EC_{50}$  (3-9  $\mu M$ ) component appeared, predominated at an  $\alpha 3$ : $\beta 2(u)$  message ratio of 1:10, and was exclusively expressed at an  $\alpha 3$ : $\beta 2(u)$  message ratio of 1:20. Without  $\beta 2$ -UTR, however, exclusive expression of the high-sensitivity  $\alpha 3\beta 2$  subform could not be achieved at a message ratio up to 1:20. Thus,  $\beta 2(u)$  appeared to regulate expression of higher-sensitivity forms of  $\alpha 3\beta 2$  as well as  $\alpha 4\beta 2$ .

### **Antagonist potency at $\alpha 4\beta 2$ subforms**

It remains unclear whether native  $\alpha 4\beta 2$  nAChR are better represented by the higher-sensitivity form, the lower sensitivity form, or whether both forms may be expressed and regulated

differentially according to cell type or maturation. However, the  $\alpha 4(u)$  and  $\beta 2(u)$  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  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$ . This was performed using receptors expressed from  $\alpha 4(u)\beta 2(u)$  to generate the high-sensitivity form alone, and from  $\alpha 4\beta 2$  without UTR to generate mixed high- and low-sensitivity receptors; we were not able to express the low-sensitivity form alone. With both  $\alpha 4(u)\beta 2(u)$  and  $\alpha 4\beta 2$ , antagonist  $IC_{50}$  values were measured against two concentrations of acetylcholine, 2  $\mu M$  (near the high-sensitivity  $EC_{50}$ ) and 200  $\mu M$  (near the low-sensitivity  $EC_{50}$ ). In the mixed-sensitivity  $\alpha 4\beta 2$  population, most (~97%) of the response to 2  $\mu M$  acetylcholine should have been from the high-sensitivity  $\alpha 4\beta 2$  subform, while for 200  $\mu M$  acetylcholine most (~81%) of the response should have been from the low-sensitivity  $\alpha 4\beta 2$  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- $\beta$ -erythroidine (DH $\beta$ E) nor methyllycaconitine distinguished between the high- and low-sensitivity forms (**Figure 7**).  $IC_{50}$  values were 3-6 nM for DH $\beta$ E and 40-135 nM for methyllycaconitine under all conditions. In contrast, chlorisondamine, and to some extent mecamlamine and *d*-tubocurarine, appeared to be selective for the low sensitivity form (**Figure**

8). Using 200  $\mu\text{M}$  acetylcholine and the mixed-sensitivity  $\alpha 4\beta 2$ , the  $\text{IC}_{50}$  values were 0.2  $\mu\text{M}$  for mecamylamine, 0.9  $\mu\text{M}$  for *d*-tubocurarine, and 0.2  $\mu\text{M}$  for chlorisondamine. Using the isolated high sensitivity form,  $\alpha 4(\text{u})\beta 2(\text{u})$ , and 2  $\mu\text{M}$  acetylcholine,  $\text{IC}_{50}$  values were 8-, 5-, and 100-fold higher for mecamylamine, *d*-tubocurarine and chlorisondamine, respectively.

### **Modulation of $\alpha 4\beta 2$ subforms by estradiol**

17 $\beta$ -estradiol is a neuroactive steroid which has been found to potentiate human  $\alpha 4\beta 2$  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  $\alpha 4(\text{u})\beta 2(\text{u})$ , 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 $\alpha 4\beta 2$ subforms**

In rat brain,  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  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  $\alpha 4$  and  $\beta 2$  messages containing UTR, or  $\alpha 4$  message lacking UTR plus  $\beta 2$  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.

In contrast, analogues of A-84543 (3-[2-((S)-pyrrolidinyl)methoxy]pyridine) (Lin et al., 2001) were found to be highly selective for the high-sensitivity  $\alpha 4\beta 2$  subform, based upon efficacy determinations using  $\alpha 4(u)\beta 2(u)$  and  $\alpha 4\beta 2$ . 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  $\alpha 4\beta 2$  expressed from UTR-containing  $\alpha 4(u)\beta 2(u)$  but appeared as if it were a partial agonist in the mixed high- and low-sensitivity populations expressed from  $\alpha 4\beta 2$  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  $\alpha 4(u)\beta 2(u)$  but seemingly partial agonists in the mixed-sensitivity  $\alpha 4\beta 2$  population. These compounds appear to selectively activate the high-sensitivity  $\alpha 4\beta 2$  response, thus producing an apparent partial response from oocytes expressing low- as well as high-sensitivity  $\alpha 4\beta 2$ .

A-163554 and A-168939 were somewhat less efficacious at  $\alpha 4\beta 2$  than anticipated from their efficacy at  $\alpha 4(u)\beta 2(u)$  and assumption of 15% high-sensitivity subform in the mixed-sensitivity  $\alpha 4\beta 2$  population. This may be due to functional differences between high-sensitivity subforms from  $\alpha 4(u)\beta 2(u)$  compared to  $\alpha 4\beta 2$ , or to variance in the relative amount of the high-sensitivity subform expressed from  $\alpha 4\beta 2$ . A-162035 appeared more efficacious than the other analogues at  $\alpha 4\beta 2$ , probably because of some activity at low-sensitivity as well as high-sensitivity  $\alpha 4\beta 2$ .

### **Agonist efficacy at native $\alpha 4\beta 2$**

A-162035 and A-168939 were used to test whether receptors similar to the high-sensitivity  $\alpha 4\beta 2$  subform could be expressed in brain. A-162035 (**Figure 14A**) and A-168939 (**Figure 14B**) each stimulated  $\alpha 4\beta 2$ -mediated  $^{86}\text{Rb}^+$  flux in mouse thalamic synaptosomes. The  $\text{EC}_{50}$  values for  $^{86}\text{Rb}^+$  flux (see figure legend) were remarkably similar to the  $\text{EC}_{50}$  values determined using ferret  $\alpha 4(\text{u})\beta 2(\text{u})$ , 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  $\mu\text{M}$  (-)-nicotine which has been shown to be selective for the high-sensitivity  $\alpha 4\beta 2$  nAChR response in mouse thalamus (Marks et al., 2004; Marks et al., 1999). **Figure 14C** also shows the thalamic synaptosome response to 10  $\mu\text{M}$  (-)-nicotine in relation to the biphasic acetylcholine concentration-response relationship. Responses to 10-100  $\mu\text{M}$  A-162035 and A-168939 were essentially completely blocked by 2  $\mu\text{M}$  DH $\beta$ E, which also has been shown to be selective for the high-sensitivity  $\alpha 4\beta 2$  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  $\alpha 4(\text{u})\beta 2(\text{u})$  expressed in oocytes. Nevertheless, the synaptosome data for A-162035 and A-168939 agree well with the oocyte  $\alpha 4(\text{u})\beta 2(\text{u})$  data in contrast to the mixed-sensitivity  $\alpha 4\beta 2$  data. Overall, the results are consistent with the idea that the high-sensitivity  $\alpha 4\beta 2$  subform is expressed in brain and that the agonists A-162035 and A-168939 selectively activate that receptor.

Higher concentrations of A-162035 ( $\geq 3 \mu\text{M}$ ) and A-168939 ( $\geq 10 \mu\text{M}$ ) 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  $\alpha 4\beta 2$  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.

## Discussion

The main findings in this study are that: (a) ferret  $\alpha 4\beta 2$  nAChR could be expressed exclusively in the high-sensitivity form only from UTR-containing message; (b) the principal determinant appears to be in the  $\beta 2$  UTR although  $\alpha 4$  UTR also may contribute; (c) a high sensitivity form of  $\alpha 3\beta 2$  also could be exclusively expressed with UTR-containing  $\beta 2$ ; (d) high- and low-sensitivity  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  subform were active at native  $\alpha 4\beta 2$  in mouse brain as well as at recombinant ferret  $\alpha 4\beta 2$ .

It has been reported that the proportion of high-sensitivity  $\alpha 4\beta 2$  could be increased by increasing the amount of  $\beta 2$  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  $\alpha 4$ - $\beta 2$  concatamer arrangement or the addition of free  $\beta 2$  message. These studies have suggested that high- and low-sensitivity components may correspond to  $\alpha 4_{(2)}\beta 2_{(3)}$  and  $\alpha 4_{(3)}\beta 2_{(2)}$  pentamers, respectively.

Using ferret messages, increasing the relative amount of  $\beta 2$  message appeared to increase the proportion of high-sensitivity  $\alpha 4\beta 2$ , similar to previous reports with  $\alpha 4\beta 2$  from other species. Zwart and Vijverberg (1998) also observed mixed high- and low-sensitivity  $\alpha 4\beta 2$ , even with the 1:9 message ratio. However, *exclusive* expression of the high-sensitivity  $\alpha 4\beta 2$  subform (or the

high-sensitivity  $\alpha 3\beta 2$  subform) could be achieved when using ferret  $\beta 2$  message containing UTR, but not when using messages lacking UTR. It is assumed that the same  $\alpha 4$  and  $\beta 2$  proteins are expressed with or without UTR. High-sensitivity ferret  $\alpha 4\beta 2$  expression may be particularly dependent upon the presence of UTR for message stability or protein translation, and at very low  $\alpha 4:\beta 2$  ratios without UTR the small amount of  $\alpha 4$  may limit the ability to detect functional  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  expression; this remains to be investigated. Additionally, it should be noted that the  $\beta 2$  TM3-TM4 cytoplasmic loop is shorter in ferret  $\beta 2$  than in human and rat  $\beta 2$ , 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,  $\alpha 4\beta 2$  or  $\alpha 3\beta 2$  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  $\alpha 4$  UTR also appeared to have an effect on exclusive expression of the high-sensitivity form. Interestingly, the 5'  $\alpha 4$  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  $\alpha 4$  5' ORF affects coding sequence translation or is itself translated.



For  $\alpha 3\beta 2$ , a wide range of acetylcholine  $EC_{50}$  values have been reported, from 1.2 to 443  $\mu M$  (Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997; Gerzanich et al., 1995) and Covernton and Connolly (2000) suggested a biphasic  $\alpha 3\beta 2$  concentration-response. Using ferret  $\beta 2$  with UTR, we demonstrated that  $\alpha 3\beta 2$  as well as  $\alpha 4\beta 2$  indeed could exhibit a biphasic concentration-response relationship for acetylcholine. Furthermore, the high-sensitivity  $\alpha 3\beta 2$  subform could be exclusively expressed using a 1:20 ratio of  $\alpha 3:\beta 2(u)$ . To our knowledge, this is the first report that decreasing  $\alpha 3:\beta 2$  message ratio influences  $\alpha 3\beta 2$  sensitivity to acetylcholine, and the first exclusive expression of the high-sensitivity subform.

In many studies with recombinant  $\alpha 4\beta 2$  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,  $\alpha 4\beta 2$  nAChR demonstrate low  $EC_{50}$  corresponding to high-sensitivity  $\alpha 4\beta 2$  (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  $\alpha 4\beta 2$ , several antagonists and agonists were evaluated for selectivity. These experiments utilized  $\alpha 4(u)\beta 2(u)$  to express exclusively the high-sensitivity subform, and  $\alpha 4\beta 2$  to express a mixture of high- and low-sensitivity subforms. While the antagonists DH $\beta$ E and methyllycaconitine were not selective between  $\alpha 4\beta 2$  subforms, chlorisondamine, mecamlamine and *d*-tubocurarine were somewhat selective for the low-sensitivity  $\alpha 4\beta 2$  subform. Our results

with *d*-tubocurarine were generally similar to those of Zwart and Vijverberg (Zwart and Vijverberg, 1998) using another species'  $\alpha 4\beta 2$ . Both studies found low  $IC_{50}$  (0.5-1  $\mu M$ ) and low Hill coefficient (0.71-0.77) for 1:1  $\alpha 4\beta 2$  and high concentrations of acetylcholine (200 or 300  $\mu M$ ), and both found similar values (2-5  $\mu M$   $IC_{50}$ s, 0.67-0.78 Hill coefficients) for high sensitivity  $\alpha 4\beta 2$  and lower concentrations of acetylcholine (2 or 10  $\mu M$ ). With 1:9  $\alpha 4\beta 2$  and 300  $\mu M$  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  $\alpha 4\beta 2$  obtained with the 1:9 ratio. In our experiments with  $\alpha 4(u)\beta 2(u)$  and 200  $\mu M$  acetylcholine or 1:1  $\alpha 4\beta 2$  and 2  $\mu M$  acetylcholine, we observed high  $IC_{50}$  values (50-100  $\mu M$ ) 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  $\alpha 4\beta 2$  may reflect differences between high- and low sensitivity  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$ , agonists displaying efficacy selective for high-sensitivity  $\alpha 4\beta 2$  could be identified. Analogs of A-84543 (Lin et al., 2001) appeared to activate predominantly high-sensitivity  $\alpha 4\beta 2$ . A-163554, A-162035 and A-168939 were full agonists at the high-sensitivity  $\alpha 4(u)\beta 2(u)$  subform. In contrast, these compounds had the appearance of partial agonists in the mixed-sensitivity  $\alpha 4\beta 2$  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.

To determine whether such compounds could activate native  $\alpha 4\beta 2$ , the effect on  $^{86}\text{Rb}^+$  flux in mouse brain thalamic synaptosomes was measured under conditions selective for the  $\alpha 4\beta 2$  component. A-162035 and A-168939 stimulated  $^{86}\text{Rb}^+$  flux to an extent nearly similar to that of 10  $\mu\text{M}$  nicotine, which has been shown to produce a near-maximal  $\alpha 4\beta 2$  effect in this assay (Marks et al., 2004; Marks et al., 1999). Indeed, the  $\text{EC}_{50}$  values for these compounds in mouse brain were similar to the values determined using high-sensitivity  $\alpha 4(\text{u})\beta 2(\text{u})$  expressed in *Xenopus* oocytes. Further, thalamic responses to A-162035 and A-168939 were blocked by the  $\alpha 4\beta 2$  antagonist DH $\beta$ E. These observations support the idea that high-sensitivity  $\alpha 4\beta 2$  represents a native  $\alpha 4\beta 2$  nAChR.

A simple assumption is that the mixed-sensitivity  $\alpha 4\beta 2$  responses resulted from expression of different  $\alpha 4\beta 2$  receptors (e.g.  $\alpha 4_{(3)}\beta 2_{(2)}$  and  $\alpha 4_{(2)}\beta 2_{(3)}$ ) with the high-sensitivity component ( $\alpha 4_{(2)}\beta 3_{(3)}$ ) corresponding to the receptor expressed from UTR-containing  $\alpha 4$  and  $\beta 2$  messages or low  $\alpha 4:\beta 2$  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  $\alpha 4(\text{u})\beta 2(\text{u})$  than  $\alpha 4\beta 2$  stimulated by 2  $\mu\text{M}$  acetylcholine even though responses were expected to be predominantly ( $\geq 97\%$ ) from the receptor with high-sensitivity to acetylcholine in both measurements. Nicotine was a partial agonist (24%) at  $\alpha 4(\text{u})\beta 2(\text{u})$ , yet it appeared to be essentially a full agonist at the high-sensitivity component of ferret mixed-sensitivity  $\alpha 4\beta 2$  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.  $\alpha$ - $\alpha$

versus  $\alpha$ - $\beta$ ), non-independent  $\alpha$ - $\beta$  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  $\alpha 4\beta 2$  and  $\alpha 3\beta 2$  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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### **Footnotes**

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### Figure Legends

**Figure 1. Ferret  $\alpha 4$  and  $\beta 2$  UTR.** The sizes of the 5' UTR (top-down diagonal) and 3' UTR (bottom-up diagonal) segments of ferret  $\alpha 4$  and  $\beta 2$  cloned from the cDNA library are diagrammed relative to the length of the coding sequences. The  $\beta 2$  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  $\alpha 4\beta 2$  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  $\mu$ M acetylcholine for high-sensitivity  $\alpha 4\beta 2$  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  $\alpha 4$  ( $\alpha 4(u)$ ) and  $\beta 2$  ( $\beta 2(u)$ ) 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  $\mu$ M (1.9-2.6  $\mu$ M c.i.), Hill slope of  $1.05 \pm 0.06$  and plateau of  $105 \pm 0.8$  %. 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  $\alpha 4$  and  $\beta 2$  nAChR messages lacking UTR. The fitted curve is the sum of two independent Hill equations with  $EC_{50}$  values of  $0.54 \mu\text{M}$  ( $0.086\text{-}3.41 \mu\text{M}$  c.i.) and  $114 \mu\text{M}$  ( $90\text{-}145 \mu\text{M}$  c.i.), Hill slopes of  $1.01 \pm 0.35$  and  $1.39 \pm 0.23$  and plateaus of  $13 \pm 4 \%$  and  $84 \pm 6 \%$ . Data are shown as mean  $\pm$  SEM or mean  $\pm$  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  $\alpha 4$  and  $\beta 2$  subunit messages in various ratios according to nucleotide content. Volume and total nucleotide content were similar. Relative amounts injected were (A) 9  $\alpha 4$  to 1  $\beta 2$ , (B) 1  $\alpha 4$  to 1  $\beta 2$ , (C) 1  $\alpha 4$  to 9  $\beta 2$ , (D) 1  $\alpha 4$  to 20  $\beta 2$ , (E) 1  $\alpha 4$  to 60  $\beta 2$  and (F) 1  $\alpha 4$  to 120  $\beta 2$ . Data for (B) are the same as in **Figure 2B** and are reproduced here to facilitate comparison with other  $\alpha 4$ : $\beta 2$  ratios. Concentration-response parameters, determined as in Figure 2, are provided in Table 1.

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

**Figure 5. Increased proportion of  $\beta 2$  message containing UTR eliminates the low-sensitivity component.** Oocytes were injected with  $\alpha 4$  and  $\beta 2$  nAChR subunit messages both lacking UTR (open symbols) or  $\alpha 4$  nAChR subunit message lacking UTR plus  $\beta 2$  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  $\beta 2$  subunit relative to  $\alpha 4$  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  $\alpha 3\beta 2$  nAChR using UTR-containing message.** Oocytes were injected with  $\alpha 3$  subunit message lacking UTR plus  $\beta 2$  subunit message either lacking UTR (open symbols) or containing 3'- and 5'-UTR (closed symbols). The  $\alpha 3:\beta 2$  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- $\beta$ -erythroidine and methyllycaconitine are not selective for high- versus low-sensitivity  $\alpha 4\beta 2$  nAChR.** Concentration-inhibition data for dihydro- $\beta$ -erythroidine (DH $\beta$ E, parts A & B) and methyllycaconitine (MLA, parts C & D) were obtained in oocytes expressing a mixture of high- and low-sensitivity  $\alpha 4\beta 2$  nAChR (open symbols) and in oocytes expressing exclusively the high-sensitivity  $\alpha 4\beta 2$  nAChR from UTR-containing  $\alpha 4$  and  $\beta 2$  messages (closed symbols). For both mixed-sensitivity and exclusively high-sensitivity nAChR, inhibition was measured against 2  $\mu$ M acetylcholine (parts A & C) and 200  $\mu$ M acetylcholine (parts B & D). Concentration-inhibition parameters are provided in Table 3.

**Figure 8. Three antagonists appear selective for the low-sensitivity  $\alpha 4\beta 2$  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  $\alpha 4\beta 2$  nAChR (open symbols) and high-sensitivity  $\alpha 4\beta 2$  nAChR from UTR-containing  $\alpha 4$  and  $\beta 2$  messages (closed symbols). For both mixed- and high-sensitivity  $\alpha 4\beta 2$ , the inhibition of responses to 2  $\mu$ M acetylcholine (**A, C & E**) and 200  $\mu$ M acetylcholine (**B, D & F**) was measured. Concentration-inhibition parameters are provided in Table 3.

**Figure 9. Estradiol potentiation of  $\alpha 4\beta 2$  nAChR.** Acetylcholine concentration-response data in the absence and presence of 10  $\mu$ M 17 $\beta$ -estradiol were obtained from three oocytes expressing high-sensitivity  $\alpha 4\beta 2$  nAChR using UTR-containing messages and from three oocytes expressing mixed-sensitivity  $\alpha 4\beta 2$  nAChR using messages lacking UTR. The concentration of acetylcholine in the reference control was 100  $\mu$ M for both mixed- and high-sensitivity  $\alpha 4\beta 2$  oocytes. **(A)** At high-sensitivity  $\alpha 4\beta 2$  nAChR, estradiol increased the maximal response by 64%. In the absence (open symbols) and presence (closed symbols) of estradiol, respectively, the acetylcholine EC<sub>50</sub> values were 2.2  $\mu$ M (1.8-2.6  $\mu$ M c.i.) and 1.7  $\mu$ M (1.0-2.7  $\mu$ M c.i.), Hill slopes were 1.10  $\pm$  0.10 and 1.10  $\pm$  0.24 and plateaus were 102  $\pm$  2 % and 166  $\pm$  8 % relative to 100  $\mu$ M 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  $\alpha 4\beta 2$  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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> 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.

**Figure 11. Selectivity of A-163554 for high-sensitivity  $\alpha 4\beta 2$ .** Responses are shown for A-163554 acting on the high-sensitivity receptor expressed from  $\alpha 4$  and  $\beta 2$  messages containing UTR ( $\alpha 4(u)\beta 2(u)$ , n=3) and the mixed-sensitivity population expressed from messages lacking UTR ( $\alpha 4\beta 2$ , n=3). As an additional control, full concentration-response relationships for acetylcholine were determined in the same three  $\alpha 4\beta 2$  oocytes exposed to A-163554. The fitted curves for A-163554 at  $\alpha 4(u)\beta 2(u)$  show an  $EC_{50}$  value of 4.8  $\mu M$  (3.1-7.3  $\mu M$  c.i.), Hill slope of  $1.34 \pm 0.28$  and plateau of  $167 \pm 12$  % relative to 1 mM acetylcholine. Using mixed-sensitivity  $\alpha 4\beta 2$ , the responses to A-163554 again were fitted well by a monophasic concentration-response curve, with an  $EC_{50}$  value of 0.74  $\mu M$  (0.23-2.4  $\mu M$  c.i.), Hill slope of  $0.85 \pm 0.30$  and plateau of  $15 \pm 2$  % relative to 1 mM acetylcholine. The same  $\alpha 4\beta 2$  oocytes demonstrated a biphasic concentration response to acetylcholine with  $EC_{50}$  values of 0.92  $\mu M$  (0.39-2.2  $\mu M$  c.i.) and 105  $\mu M$  (92-119  $\mu M$  c.i.), Hill slopes of  $1.05 \pm 0.26$  and  $1.35 \pm 0.13$ , and plateaus of  $19 \pm 3$  % and  $85 \pm 5$  % relative to 1 mM acetylcholine.

**Figure 12. Selectivity of A-162035 for high-sensitivity  $\alpha 4\beta 2$ .** Responses are shown for A-162035 acting on the high-sensitivity receptor expressed from  $\alpha 4$  and  $\beta 2$  messages containing UTR ( $\alpha 4(u)\beta 2(u)$ , n=3) and the mixed-sensitivity population expressed from messages lacking UTR ( $\alpha 4\beta 2$ , n=4). The fitted curves for A-162035 at  $\alpha 4(u)\beta 2(u)$  show an  $EC_{50}$  value of 0.13  $\mu M$  (0.11-0.16  $\mu M$  c.i.), Hill slope of  $1.49 \pm 0.20$  and plateau of  $113 \pm 3$  % relative to 1 mM acetylcholine. Using  $\alpha 4\beta 2$  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  $\mu M$



(0.02-108  $\mu\text{M}$  c.i.), Hill slope of  $0.57 \pm 0.35$  and plateau of  $41 \pm 26$  % relative to 1 mM acetylcholine.

**Figure 13. Selectivity of A-168939 for high-sensitivity  $\alpha 4\beta 2$ .** Responses are shown for A-168939 acting on the high-sensitivity receptor expressed from  $\alpha 4$  and  $\beta 2$  messages containing UTR ( $\alpha 4(u)\beta 2(u)$ , n=6) and the mixed-sensitivity population expressed from messages lacking UTR ( $\alpha 4\beta 2$ , n=6). The fitted curve for A-168939 at  $\alpha 4(u)\beta 2(u)$  shows an  $\text{EC}_{50}$  value of 0.71  $\mu\text{M}$  (0.54-0.93  $\mu\text{M}$  c.i.), Hill slope of  $1.31 \pm 0.18$  and plateau of  $86 \pm 3$  % relative to 1 mM acetylcholine. A-168939 had little effect at  $\alpha 4\beta 2$  mixed-sensitivity receptors. The fitted curve reflects an  $\text{EC}_{50}$  value of 7.5  $\mu\text{M}$  (0.16-360  $\mu\text{M}$  c.i.), Hill slope of  $0.65 \pm 0.40$  and plateau of  $8 \pm 4$  % 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  $\alpha 4\beta 2$  nAChR by A-162035 and A-168939.** Mouse thalamic synaptosome  $\text{Rb}^+$  flux was measured in response to A-162035, A-168939 and acetylcholine in the absence and presence of DH $\beta$ E. Graphs also show the response to 10  $\mu\text{M}$  (-)-nicotine (solid diamond) measured in each experiment as a positive control for high-sensitivity  $\alpha 4\beta 2$ . **(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  $\mu\text{M}$  DH $\beta$ E. The fitted curve for A-162035 shows an  $\text{EC}_{50}$  value of 0.12  $\mu\text{M}$  (0.039-0.34  $\mu\text{M}$  c.i.), Hill slope of  $1.00 \pm 0.33$ , and plateau of  $8 \pm 2$  % 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  $\mu\text{M}$

DH $\beta$ E. The fitted curve for A-168939 shows an EC<sub>50</sub> value of 0.24  $\mu$ M (0.11-0.50  $\mu$ M c.i.), Hill slope of  $1.00 \pm 0.24$ , and plateau of  $10 \pm 1$  % 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  $\mu$ M DH $\beta$ E. 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 DH $\beta$ E. The fitted curve shows a high-sensitivity component with EC<sub>50</sub> value of 0.77  $\mu$ M (0.24-2.4  $\mu$ M c.i.), Hill slope of  $1.06 \pm 0.40$ , and plateau of  $18 \pm 4$  % and a low-sensitivity component with EC<sub>50</sub> value of 57  $\mu$ M (28-115  $\mu$ M c.i.), Hill slope of  $2.00 \pm 0.97$ , and plateau of  $16 \pm 4$  %.

**Table 1. Isolation of a high-sensitivity  $\alpha 4\beta 2$  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  $\pm$  SEM. For  $\alpha 4(u)\beta 2(u)$  1:1 and  $\alpha 4\beta 2(u)$  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.

Receptor <sup>a</sup>	Ratio $\alpha:\beta$	N	acetylcholine high-sensitivity			acetylcholine low-sensitivity	
			$EC_{50}$ , $\mu M$	nH	proportion	$EC_{50}$ , $\mu M$	nH
$\alpha 4(u)\beta 2(u)$	1:1	30	2.2 (1.9-2.6)	1.05 $\pm$ 0.06	100%	--	--
$\alpha 4\beta 2$	9:1	5	0.70 (0.4-1.2)	1.5 $\pm$ 0.3	15%	130 (80-190)	1.2 $\pm$ 0.1
$\alpha 4\beta 2$	1:1	7	0.54 (0.09-3.4)	1.0 $\pm$ 0.4	14%	114 (90-145)	1.4 $\pm$ 0.2
$\alpha 4\beta 2$	1:9	6	2.3 (0.41-13)	1.2 $\pm$ 0.3	35%	120 (56-270)	1.4 $\pm$ 0.8
$\alpha 4\beta 2$	1:20	3	1.2 (0.05-30)	0.9 $\pm$ 0.7	32%	200 (71-540)	1.1 $\pm$ 0.6

Receptor <sup>a</sup>	Ratio $\alpha$ : $\beta$	N	acetylcholine high-sensitivity			acetylcholine low-sensitivity	
			EC <sub>50</sub> , $\mu$ M	nH	proportion	EC <sub>50</sub> , $\mu$ M	nH
$\alpha$ 4 $\beta$ 2	1:60	9	3.1 (1.1-8.4)	1.0 $\pm$ 0.3	38%	160 (120-210)	1.7 $\pm$ 0.3
$\alpha$ 4 $\beta$ 2	1:120	5	2.0 (0.41-9.5)	1.1 $\pm$ 0.5	46%	100 (50-210)	1.6 $\pm$ 0.8
$\alpha$ 4 $\beta$ 2	1:1	3	2.5 (0.22-29)	0.9 $\pm$ 0.5	24%	140 (110-180)	2.0 $\pm$ 0.4
$\alpha$ 4(u) $\beta$ 2	1:1	4	4.0 (0.30-55)	1.1 $\pm$ 0.6	21%	200 (120-360)	1.5 $\pm$ 0.6
$\alpha$ 4 $\beta$ 2(u)	1:1	10	0.85 (0.16-4.6)	1.3 $\pm$ 1.1	39%	120 (45-350)	1.1 $\pm$ 0.6
$\alpha$ 4(u) $\beta$ 2(u)	1:1	3	2.1 (2.0-2.2)	1.15 $\pm$ 0.02	100%	--	--
$\alpha$ 4 $\beta$ 2	1:1	4	1.5 (0.56-4.2)	1.1 $\pm$ 0.4	16%	180 (160-210)	1.5 $\pm$ 0.2
$\alpha$ 4 $\beta$ 2(u)	1:5	4	1.6 (1.2-2.3)	1.2 $\pm$ 0.2	100%	--	--

<sup>a</sup> Receptors were expressed from  $\alpha$ 4 and  $\beta$ 2 messages lacking UTR and from other messages, designated  $\alpha$ 4(u) and  $\beta$ 2(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  $\alpha 3\beta 2$ .**

For  $\alpha 3\beta 2(u)$  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  $\alpha 3\beta 2$  1:20 were constrained in order to fit the data.

Receptor <sup>a</sup>	Ratio $\alpha:\beta$	N	acetylcholine high-sensitivity			acetylcholine low-sensitivity	
			EC <sub>50</sub> , $\mu$ M	nH	proportion	EC <sub>50</sub> , $\mu$ M	nH
$\alpha 3\beta 2$	1:1	15	25 (7-88)	1.2 $\pm$ 0.2	15%	450 (380-540)	1.3 $\pm$ 0.1
$\alpha 3\beta 2$	1:20	6	23 (7-81)	1.2	34%	480 (200-1100)	1.2
$\alpha 3\beta 2(u)$	1:1	13	8.1 (7.1-9.1)	0.89 $\pm$ 0.03	77%	1700 (1300-2100)	2.6 $\pm$ 0.3
$\alpha 3\beta 2(u)$	1:5	3	2.8 (1.4-5.6)	1.5 $\pm$ 0.4	52%	88 (29-260)	1.1 $\pm$ 0.6
$\alpha 3\beta 2(u)$	1:10	9	9.4 (6.6-13)	1.0 $\pm$ 0.2	100%	--	--
$\alpha 3\beta 2(u)$	1:20	8	8.6 (6.0-12)	0.98 $\pm$ 0.14	100%	--	--

<sup>a</sup> Receptors were expressed from  $\alpha 3$  message lacking UTR and from  $\beta 2$  messages lacking ( $\beta 2$ ) or containing ( $\beta 2(u)$ ) 3' and 5' UTR segments.

**Table 3. Antagonist potencies at ferret  $\alpha 4\beta 2$  and at the high-sensitivity form expressed from UTR-containing  $\alpha 4(u)$  and  $\beta 2(u)$ .**

Concentration-inhibition curves projected to 100% inhibition for methyllycaconitine (MLA), mecamlamine (Mec), *d*-tubocurarine (dTC) and chlorisondamine (CI), but not for dihydro- $\beta$ -erythroidine (DH $\beta$ E). For DH $\beta$ E acting on  $\alpha 4\beta 2$  the projected maximal inhibition was  $80 \pm 3$  % with 2  $\mu$ M acetylcholine and  $95 \pm 4$  % with 200  $\mu$ M acetylcholine while these values at  $\alpha 4(u)\beta 2(u)$  were  $90 \pm 2$  % with 2  $\mu$ M acetylcholine and  $16 \pm 3$  % with 200  $\mu$ M acetylcholine.

[acetylcholine]	antagonist	$\alpha 4\beta 2$			$\alpha 4(u)\beta 2(u)$		
		IC <sub>50</sub> , $\mu$ M	nH	N	IC <sub>50</sub> , $\mu$ M	nH	N
2 $\mu$ M	DH $\beta$ E	0.0023 (0.0010-0.0050)	$0.83 \pm 0.20$	3	0.0027 (0.0020-0.0036)	$1.20 \pm 0.18$	3
	MLA	0.063 (0.049-0.081)	$1.29 \pm 0.18$	3	0.13 (0.11-0.16)	$1.52 \pm 0.18$	3
	Mec	0.58 (0.27-1.26)	$0.65 \pm 0.14$	3	2.0 (1.2-3.3)	$0.72 \pm 0.12$	3
	dTC	110 (11-1200)	$0.41 \pm 0.30$	3	4.7 (3.2-7.0)	$0.78 \pm 0.11$	4
	CI	0.52 (0.34-0.80)	$0.63 \pm 0.08$	5	18 (13-26)	$1.00 \pm 0.16$	5

[acetylcholine]	antagonist	$\alpha 4\beta 2$			$\alpha 4(u)\beta 2(u)$		
		IC <sub>50</sub> , $\mu\text{M}$	nH	N	IC <sub>50</sub> , $\mu\text{M}$	nH	N
200 $\mu\text{M}$	DH $\beta$ E	0.0056 (0.0021-0.015)	0.60 $\pm$ 0.15	3	0.0036 (0.0020-0.0062)	0.95 $\pm$ 0.21	3
	MLA	0.038 (0.032-0.045)	1.23 $\pm$ 0.11	3	0.101 (0.073-0.139)	1.23 $\pm$ 0.21	3
	Mec	0.24 (0.21-0.28)	0.90 $\pm$ 0.05	6	3.81 (2.67-5.64)	1.10 $\pm$ 0.21	6
	dTC	0.92 (0.69-1.23)	0.75 $\pm$ 0.07	5	49.7 (8.8-280)	0.39 $\pm$ 0.13	3
	CI	0.18 (0.17-0.20)	1.14 $\pm$ 0.05	6	3.3 (2.9-3.7)	0.84 $\pm$ 0.03	2

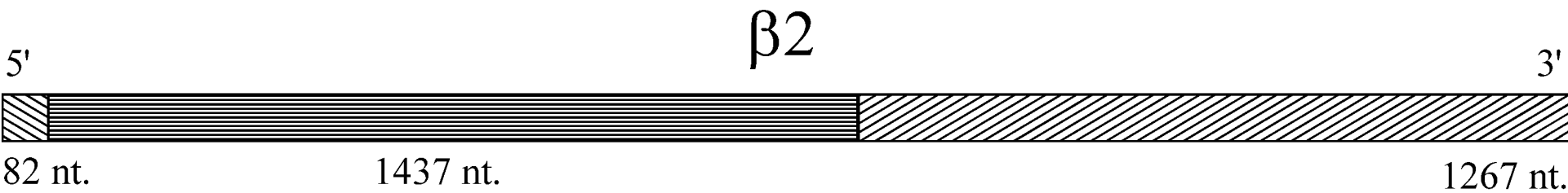
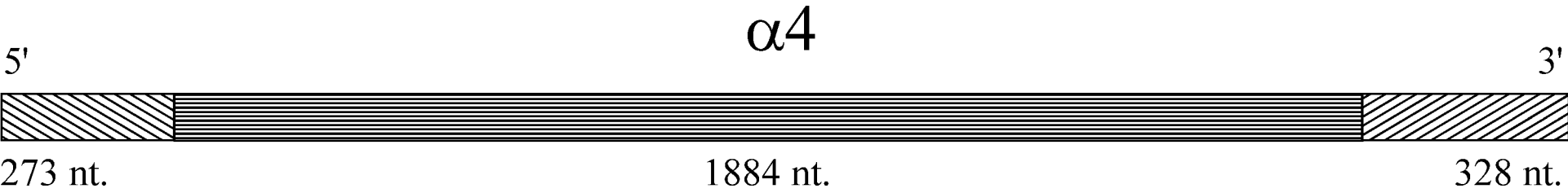
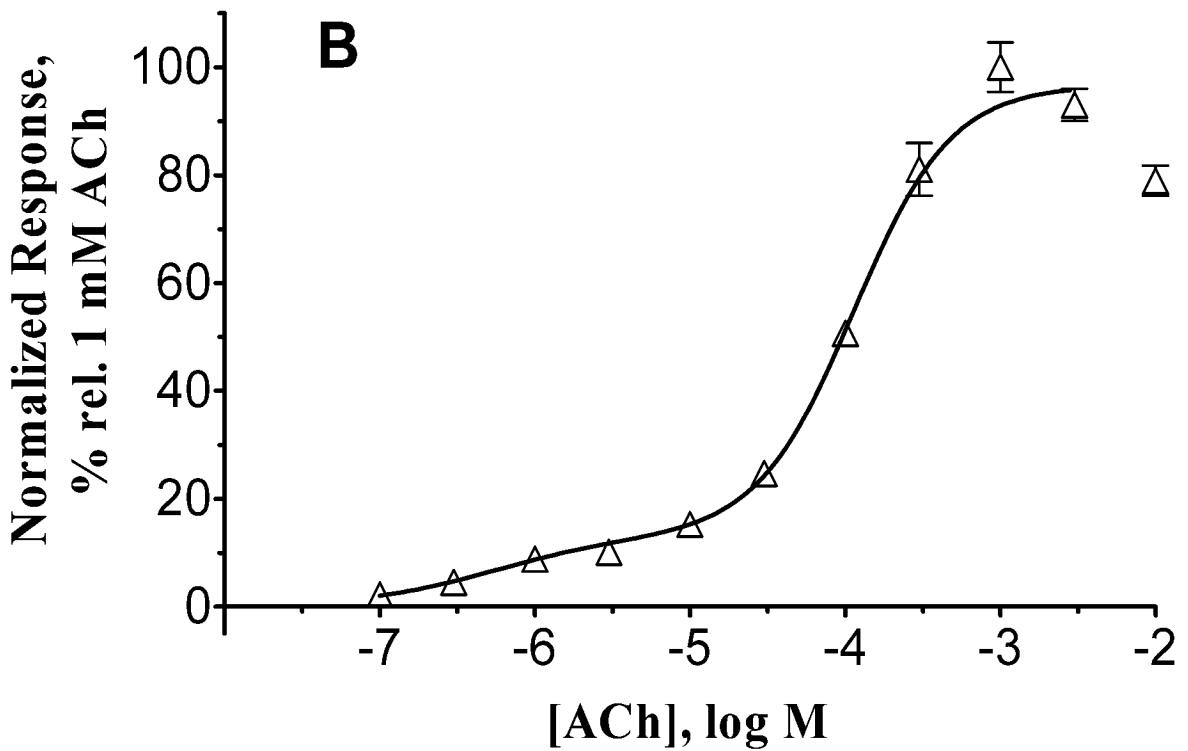
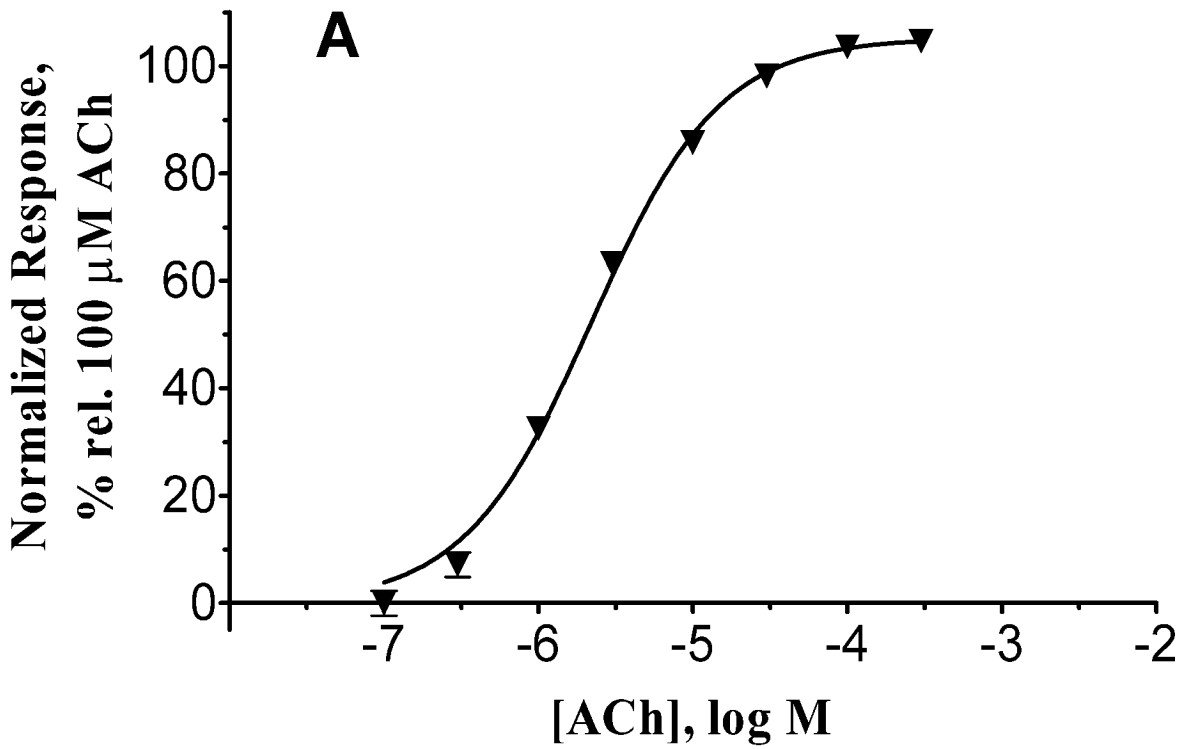
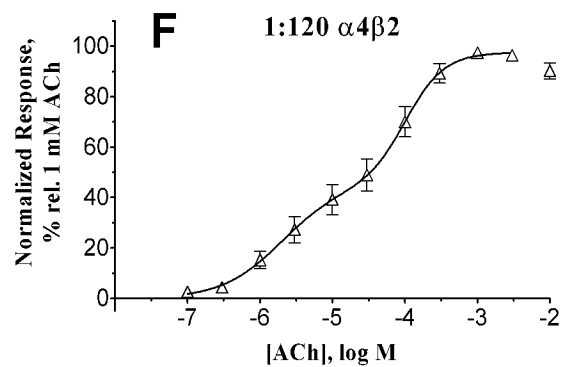
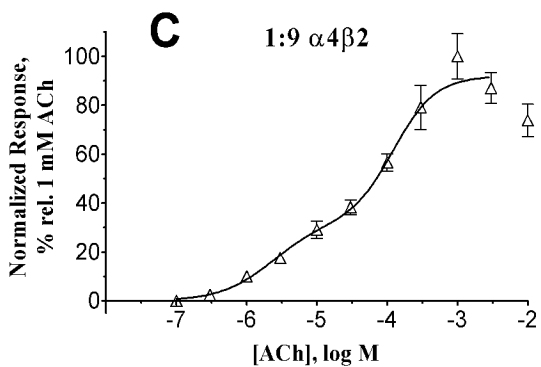
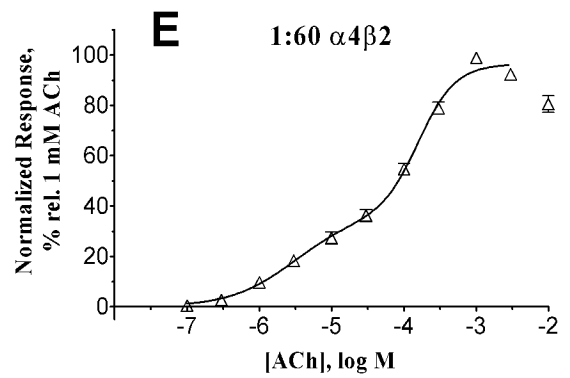
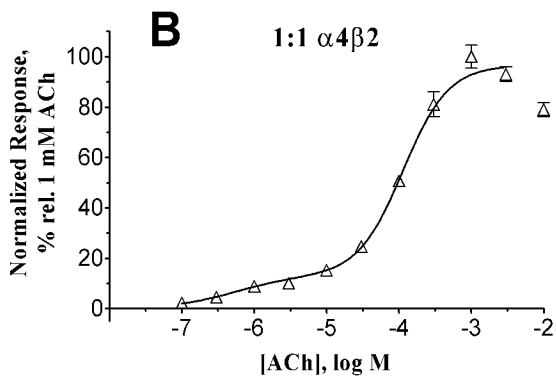
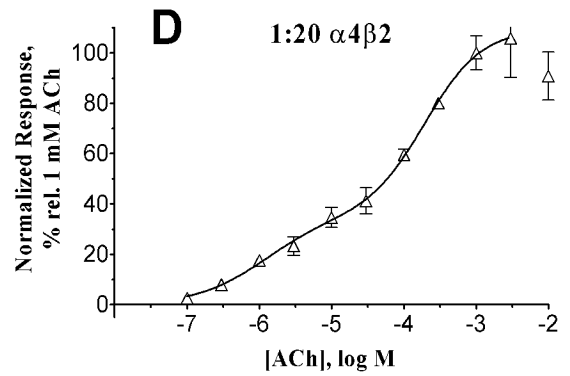
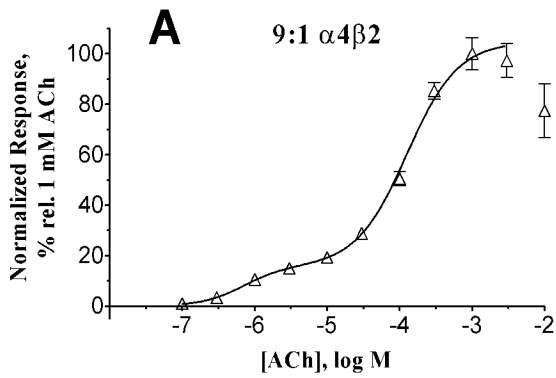




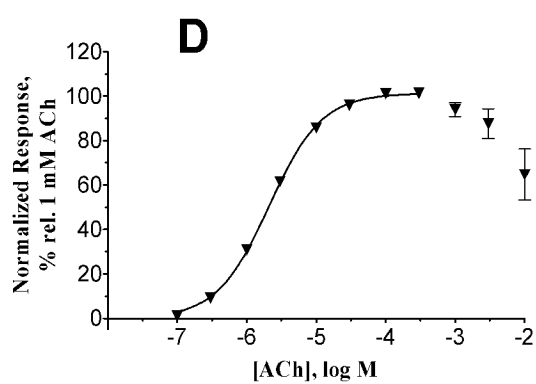
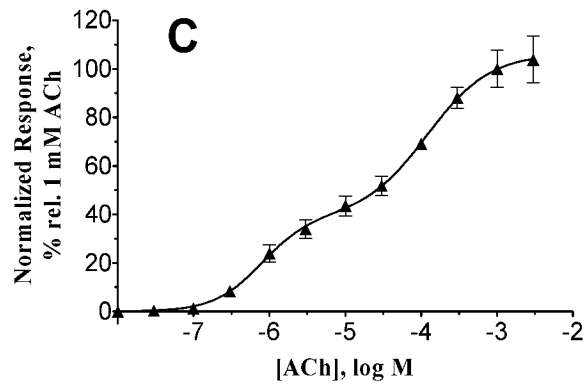
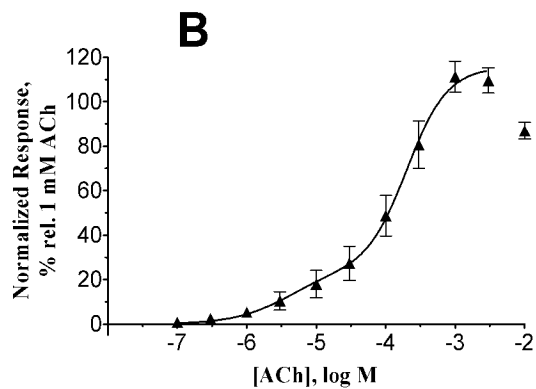
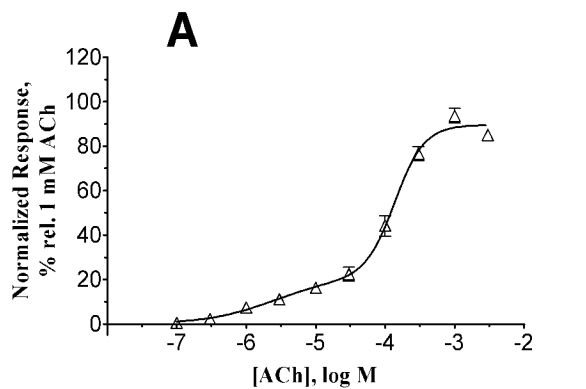
Figure 2

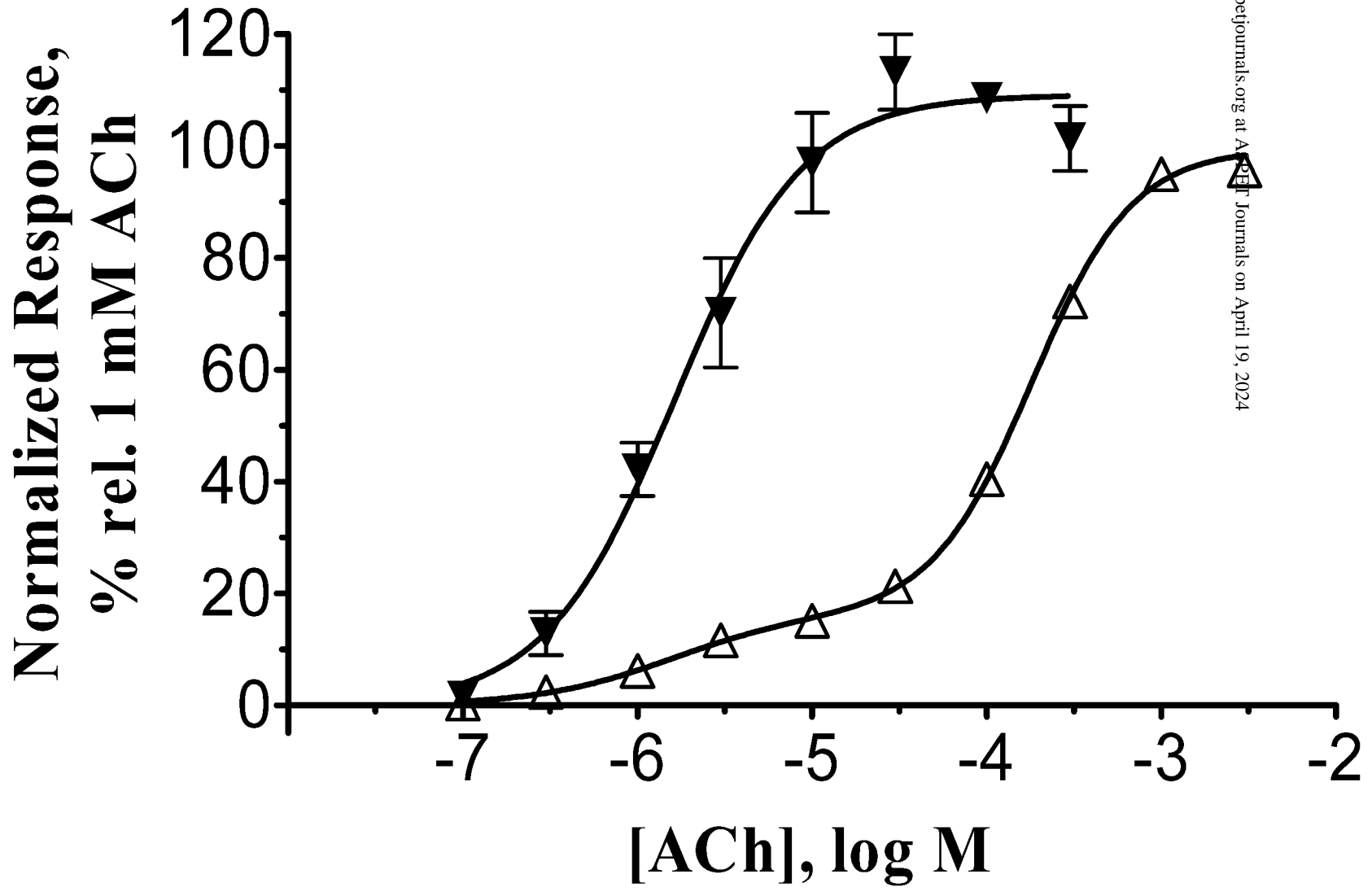


# Figure 3



# Figure 4





# Figure 6

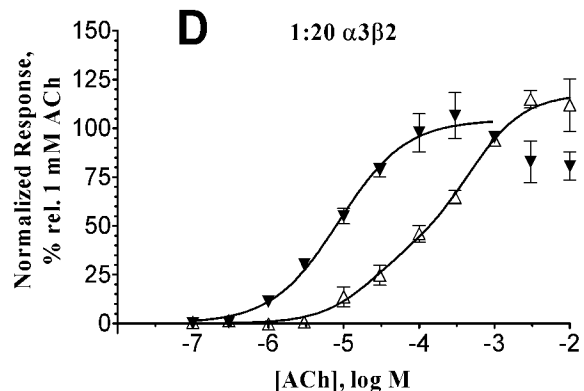
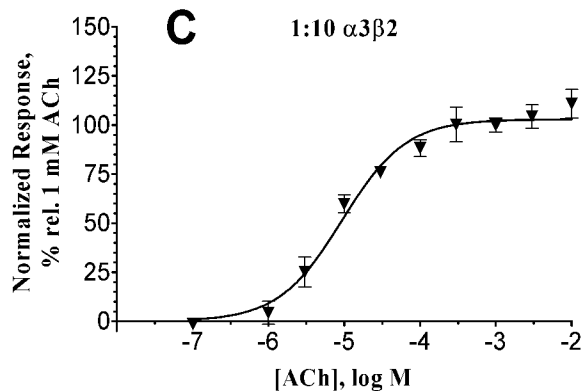
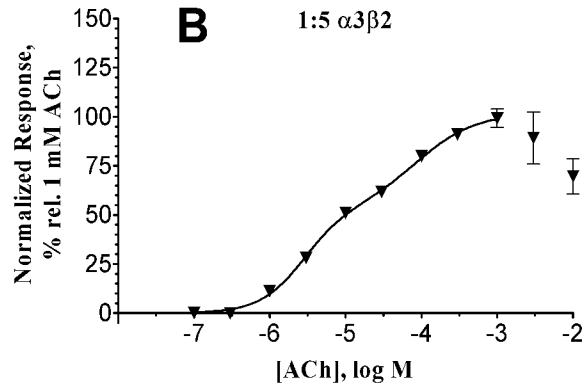
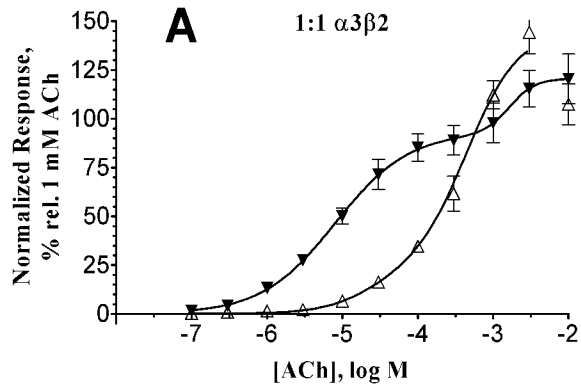
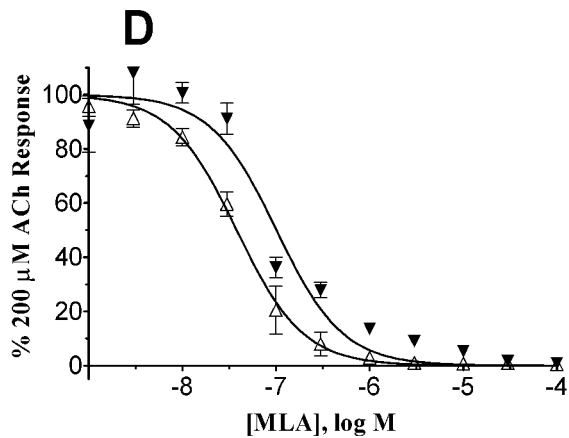
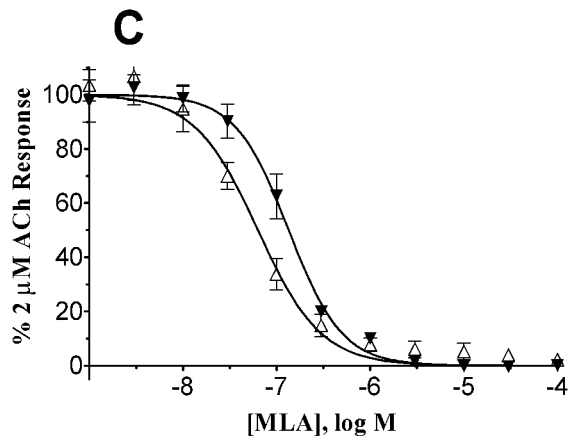
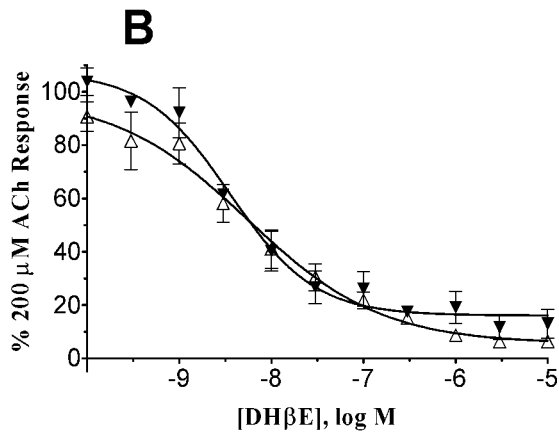
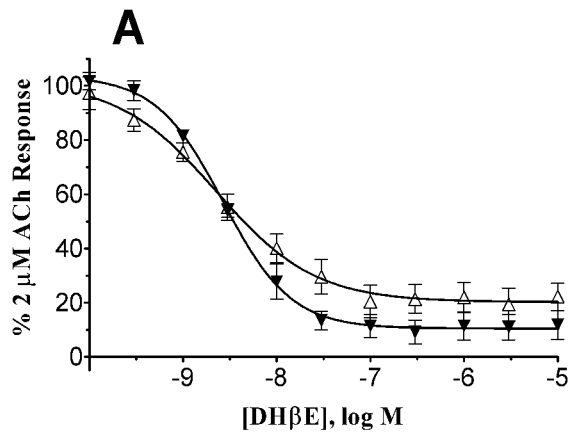


Figure 7



# Figure 8

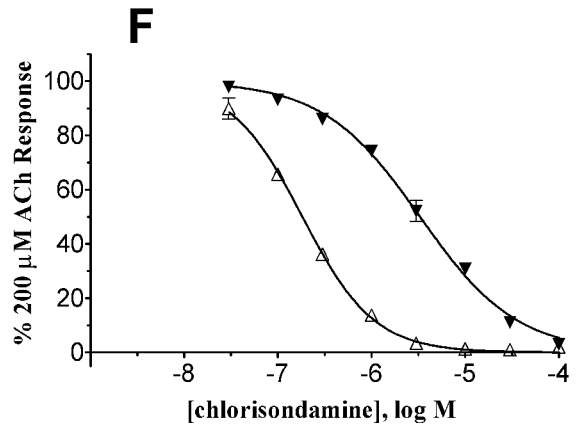
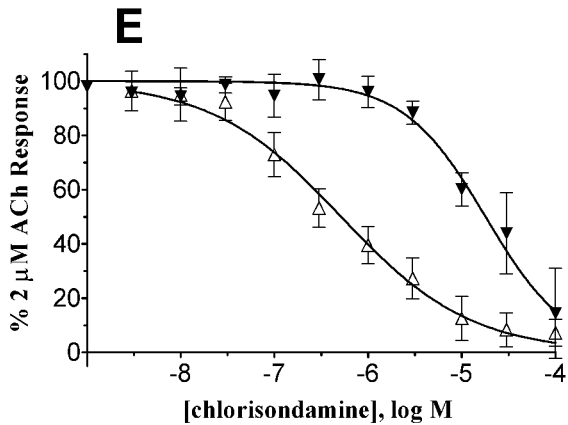
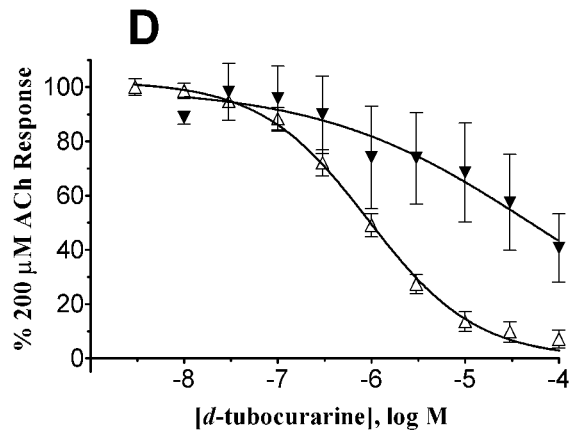
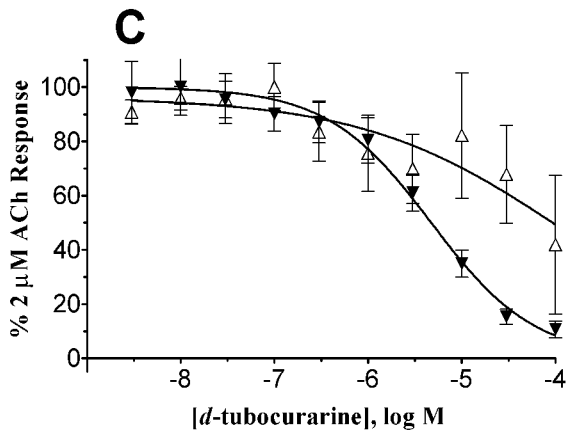
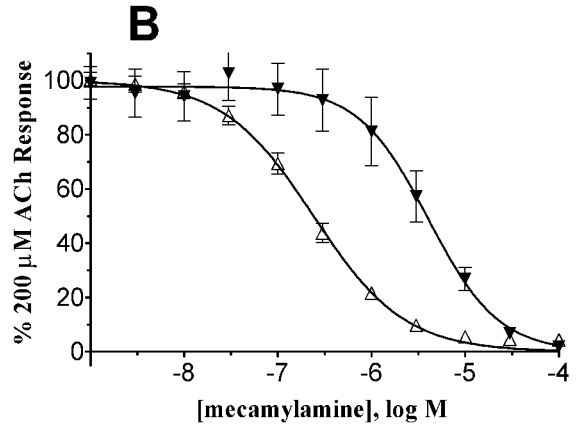
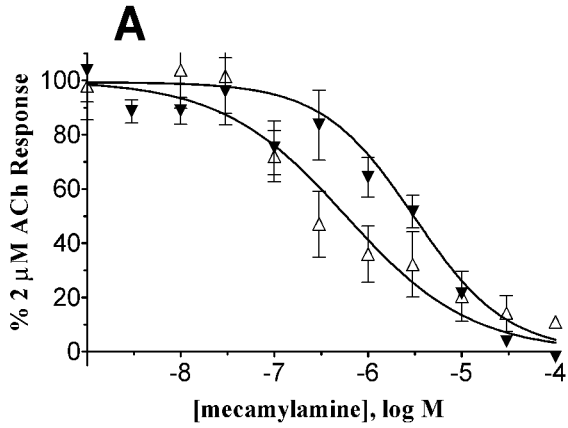
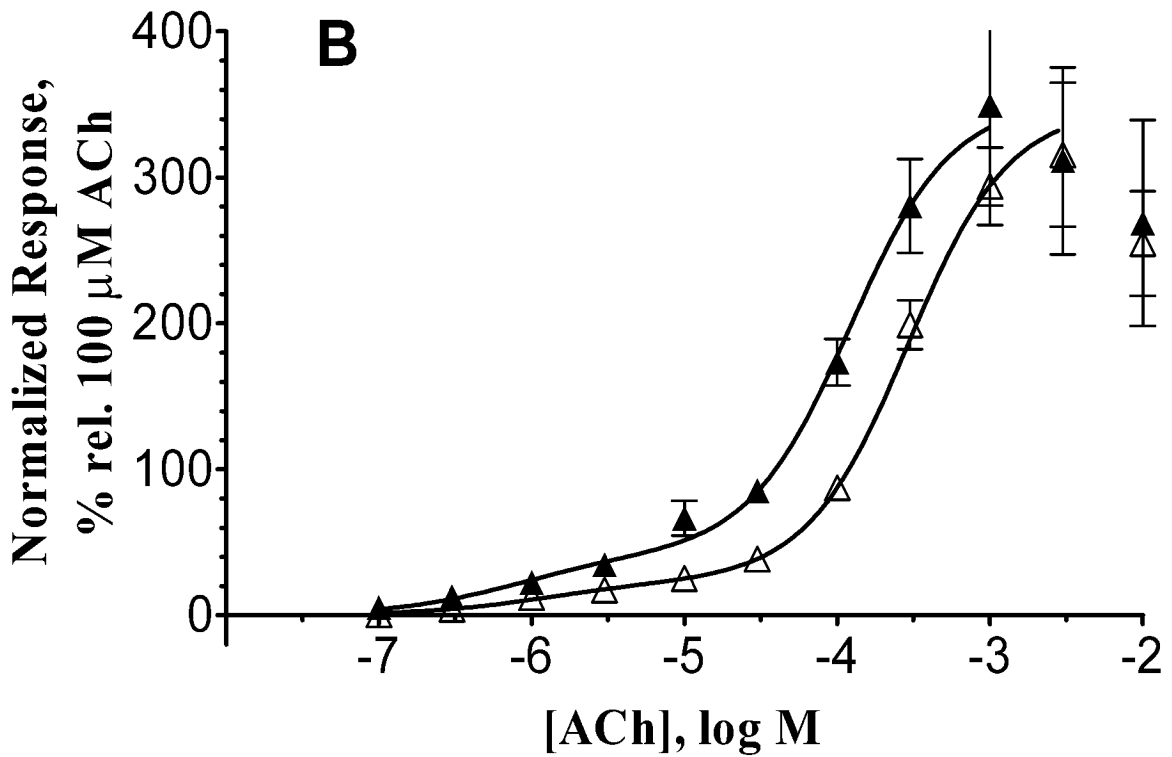
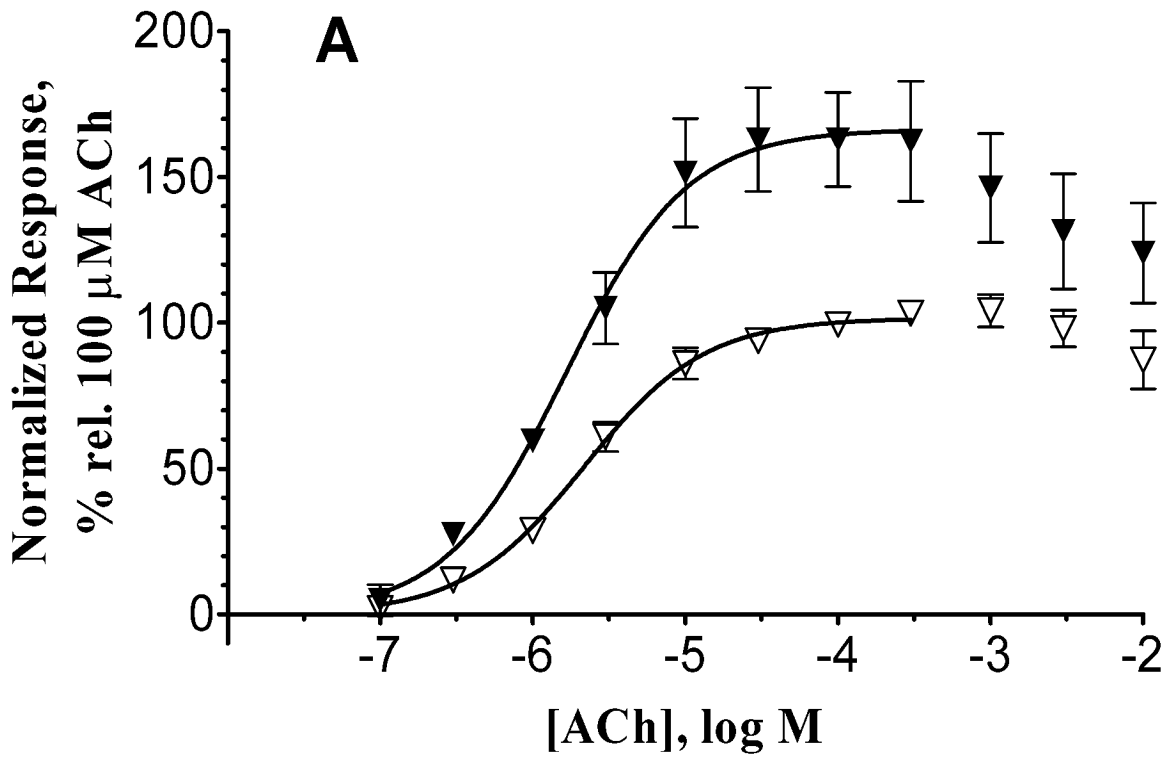


Figure 9





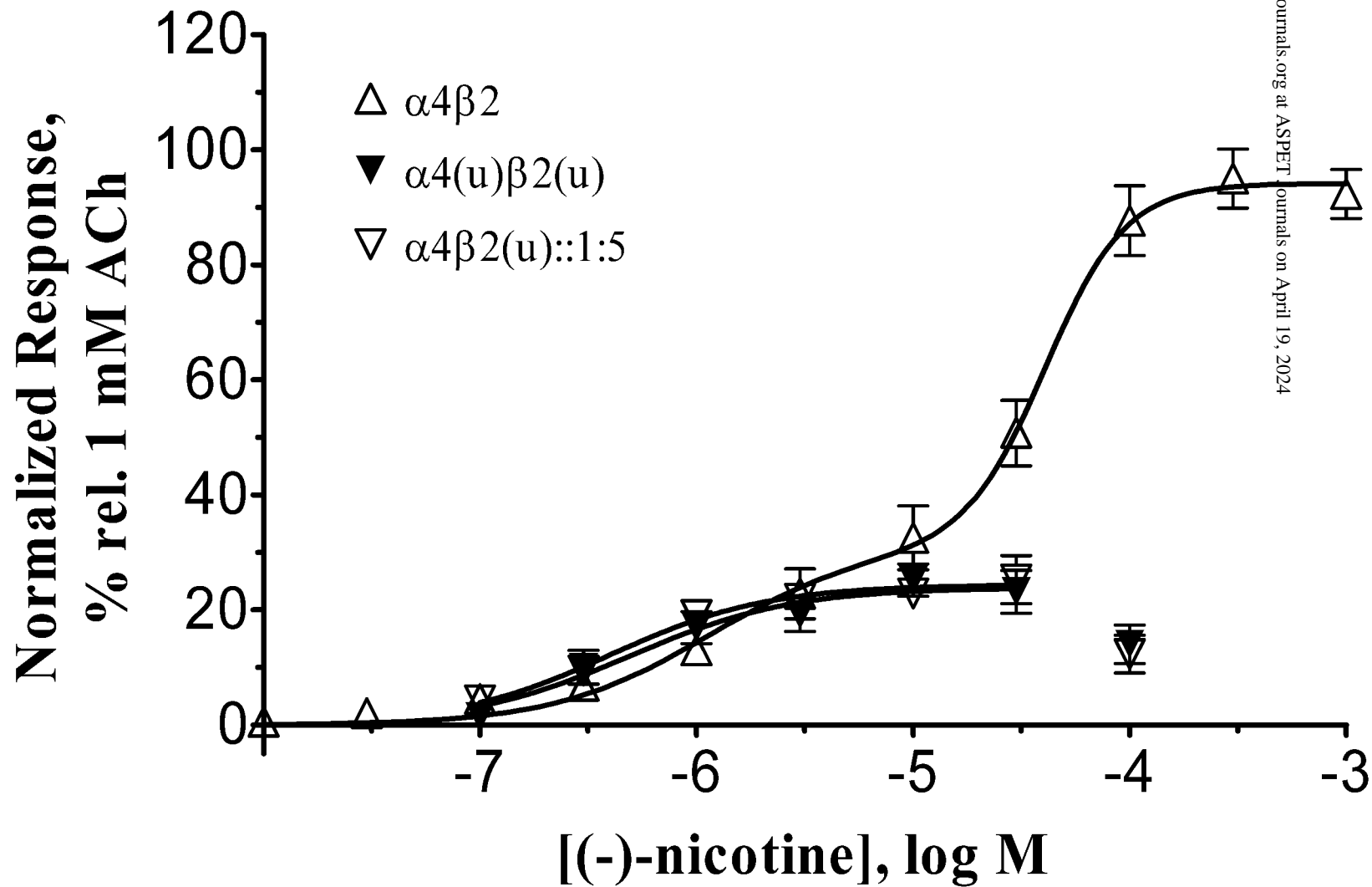
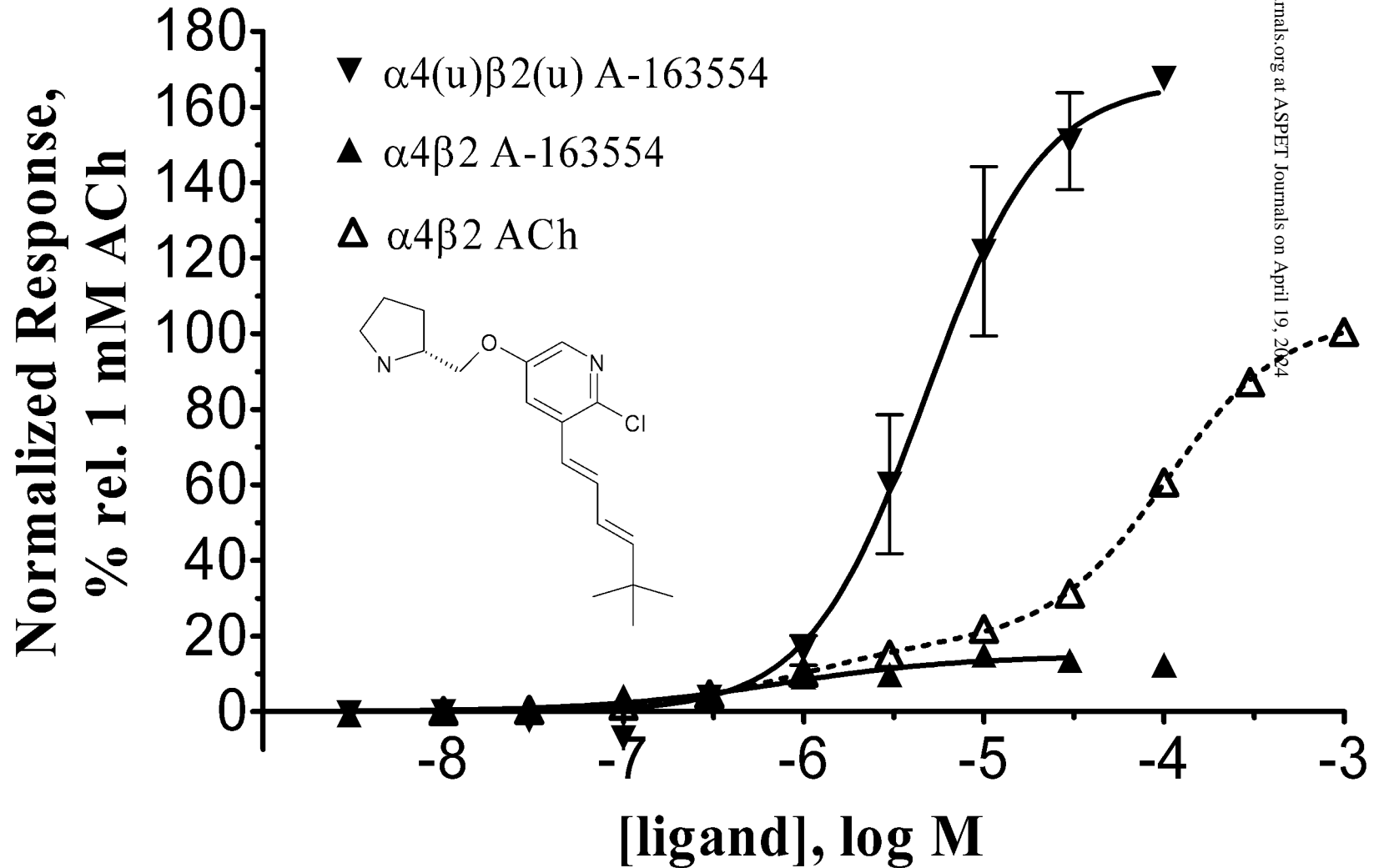


Figure 11



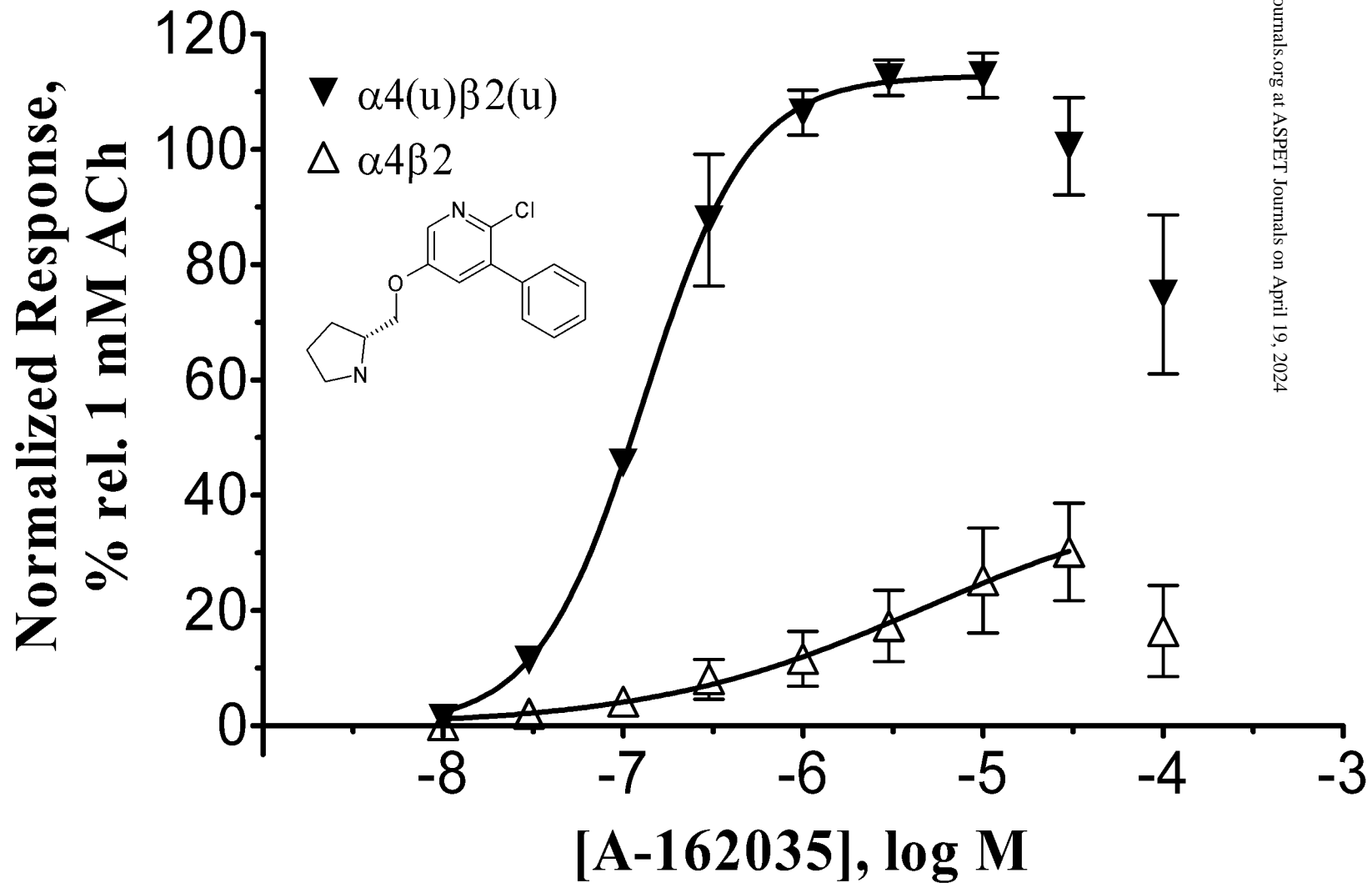
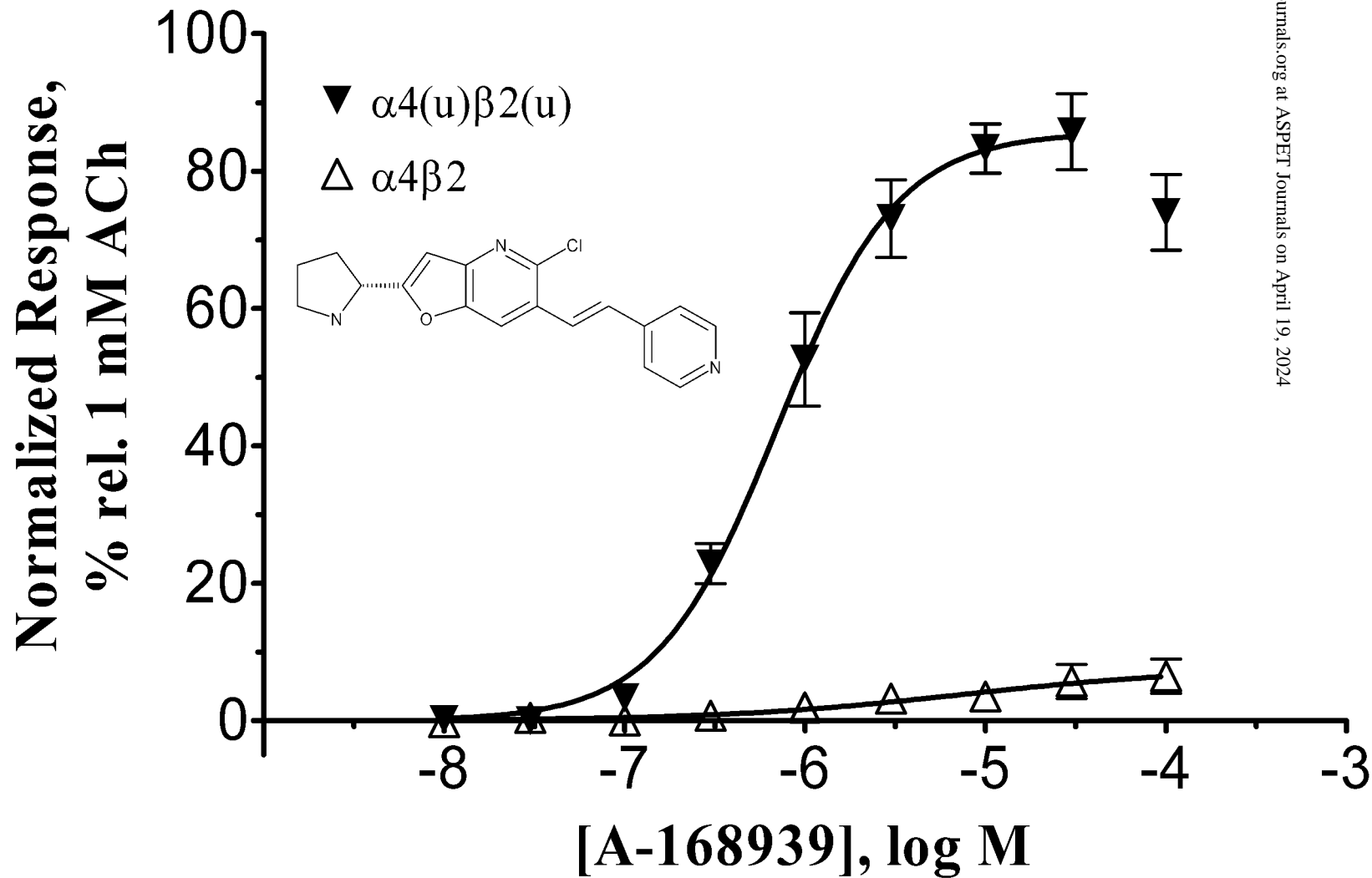


Figure 12



# Figure 14

