

$\alpha 4\beta 2$ Nicotinic Receptors with High and Low Acetylcholine Sensitivity: Pharmacology, Stoichiometry, and Sensitivity to Long-Term Exposure to Nicotine

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ABSTRACT

$\alpha 4$ and $\beta 2$ nicotinic acetylcholine receptor (nAChR) subunits expressed heterologously assemble into receptors with high (HS) and low (LS) sensitivity to acetylcholine (ACh); their relative proportions depend on the $\alpha 4$ to $\beta 2$ ratio. In this study, injection of oocytes with 1:10 $\alpha 4/\beta 2$ subunit cDNA ratios favored expression of HS $\alpha 4\beta 2$ nAChRs, as evidenced by monophasic ACh concentration-response curves, whereas injections with 10:1 cDNA ratios favored expression of LS $\alpha 4\beta 2$ receptors. The stoichiometry was inferred from the shifts in the ACh EC_{50} values caused by Leu to Thr mutations at position 9' of the second transmembrane domain of $\alpha 4$ and $\beta 2$. The 1:10 injection ratio produced the $(\alpha 4)_2(\beta 2)_3$ stoichiometry, whereas 10:1 injections produced the $(\alpha 4)_3(\beta 2)_2$ stoichiometry. The agonists epibatidine, 3-[2(S)-azetidinylmethoxy]pyridine (A-85380), 5-ethoxy-metanicotine (TC-2559), cytisine, and 3-Br-cytisine

and the antagonists dihydro- β -erythroidine and *d*-tubocurarine were more potent at HS receptors. TC-2559 was more efficacious than ACh at HS receptors but was a partial agonist at LS receptors. Epibatidine was more efficacious than ACh at LS receptors and a partial agonist at HS receptors. Cytisine and 5-halogenated cytisines had moderate efficacy at LS receptors but had almost no efficacy at HS receptors. By exploiting the differential effects of ACh, TC-2559 and 5-I-cytisine we evaluated the effects of long-term exposure to nicotine on HS and LS receptors expressed in *Xenopus laevis* oocytes after cDNA injections or microtransplantation of $\alpha 4\beta 2$ receptors assembled in human embryonic kidney 293 cells. We conclude that nicotine up-regulates HS $\alpha 4\beta 2$ receptors, probably by influencing the assembly of receptors rather than by altering the functional state of LS $\alpha 4\beta 2$ nAChRs.

The $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChR) is the most abundant nAChR expressed in mammalian brain, where it forms the high-affinity binding site for nicotine (Picciotto et al., 2001). $\alpha 4\beta 2$ nAChRs have been implicated in cognition and nociception (Picciotto et al., 2001) and Alzheimer's and Parkinson's diseases (Zanardi et al., 2002), and they play a direct role in nicotine addiction (Picciotto et al., 2001; Tapper et al., 2004; Maskos et al., 2005). Mutations in both the $\alpha 4$ (*CHRNA4*) and $\beta 2$ (*CHRNA2*) subunit genes cause autosomal nocturnal frontal lobe epilepsy (Weiland et al., 2000).

The stoichiometry of human $\alpha 4\beta 2$ nAChRs in neurons has

not yet been elucidated. Biochemical (Anand et al., 1991) and electrophysiological (Cooper et al., 1991) approaches have shown that chick $\alpha 4\beta 2$ nAChRs assemble in a stoichiometry of $(\alpha 4)_2(\beta 2)_3$ when expressed heterologously in *Xenopus laevis* oocytes, and it has been proposed that this stoichiometry predominates in the chick brain (Anand et al., 1991; Cooper et al., 1991). Several lines of evidence suggest that $\alpha 4\beta 2$ nAChR exists in two different stoichiometries in the mammalian brain. First, functional studies have shown biphasic agonist concentration-response curves for stimulation of putative $\alpha 4\beta 2$ nAChR function in mouse thalamic preparations (Marks et al., 1999; Butt et al., 2002). Such function is not present in the thalamus of $\alpha 4^{-/-}$ or $\beta 2^{-/-}$ knockout mice, which also lack high-affinity epibatidine sites (Picciotto et al., 2001). Second, $\alpha 4$ and $\beta 2$ nAChR subunits expressed heterologously in mammalian cell lines (Buisson and Bertrand, 2001; Nelson et al., 2003; Kuryatov et al., 2005; Vallejo et al.,

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; HEK, human embryonic kidney; TM2, second transmembrane domain; ACh, acetylcholine; DH β E, dihydro- β -erythroidine; CI, confidence interval; HS $\alpha 4\beta 2$ nAChR, high-sensitivity $\alpha 4\beta 2$ nAChR; LS $\alpha 4\beta 2$ nAChR, low-sensitivity $\alpha 4\beta 2$ nAChR; I_{SPT} , current through spontaneous channel openings; A-85380, 3-[2(S)-azetidinylmethoxy]pyridine; TC-2559, 5-ethoxy-metanicotine.

2005) or *X. laevis* oocytes (Zwart and Vijverberg, 1998; Zwart et al., 2006) assemble into a mixture of receptors with high and low agonist sensitivity. Functional studies in combination with quantification of methionine-labeled subunits have shown that in stably transfected HEK293 cells expressing human $\alpha 4\beta 2$ nAChR, the $\alpha 4\beta 2$ receptor with high agonist sensitivity has a stoichiometry of $(\alpha 4)_2(\beta 2)_3$ whereas the lower sensitivity receptor has a stoichiometry of $(\alpha 4)_3(\beta 2)_2$ (Nelson et al., 2003). In these cells, the $(\alpha 4)_3(\beta 2)_2$ stoichiometry predominates, but long-term exposure to nicotine (Buisson and Bertrand, 2001; Nelson et al., 2003; Kuryatov et al., 2005; Sallette et al., 2005; Vallejo et al., 2005), or boosting the number of $\beta 2$ subunits by additional transfection or culturing cells at 29°C (Nelson et al., 2003), promotes expression of the high ACh sensitivity $(\alpha 4)_2(\beta 2)_3$ receptor.

Variation of the $\alpha 4/\beta 2$ ratio injected into oocytes produces a multiplicity of $\alpha 4\beta 2$ nAChR types that cannot be accounted for by the assembly of $\alpha 4$ and $\beta 2$ subunits into only the $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ stoichiometries (Zwart and Vijverberg, 1998; López-Hernández et al., 2004). Such subtype diversity could explain discrepancies in reports of the pharmacological profile of heterologously expressed $\alpha 4\beta 2$ nAChRs; for example, nicotine has been reported to be a full (Rush et al., 2002) and a partial (Chávez-Noriega et al., 1997) agonist at human $\alpha 4\beta 2$ nAChR expressed heterologously in *X. laevis* oocytes. It is clear that if properties of heterologously expressed receptors are to be used to facilitate the identification of native $\alpha 4\beta 2$ nAChR subtypes or in drug discovery, it is crucially important to use experimental designs that favor the expression of homogeneous populations of $\alpha 4\beta 2$ nAChR types.

The objective of this work was to heterologously express, in *X. laevis* oocytes, homogeneous populations of human $\alpha 4\beta 2$ nAChRs to permit pharmacological comparison in native mammalian neurons and to examine the functional effects of long-term exposure to nicotine on specific functional types of the $\alpha 4\beta 2$ nAChR. In this study, we showed that human $\alpha 4\beta 2$ nAChRs expressed in *X. laevis* oocytes injected with either 1:10 or 10:1 $\alpha 4/\beta 2$ ratios of cDNAs displayed high or low sensitivity to ACh, respectively. We inserted a hydrophobic to hydrophilic reporter mutation in the middle of the second transmembrane domain (TM2; L9'T as numbered by Miller, 1989) of both subunits to determine the number of each subunit present in the high and low sensitivity $\alpha 4\beta 2$ receptors. Hydrophobic-to-hydrophilic mutations at 9' (Labarca et al., 1995; Boorman et al., 2000) or other positions (Plazas et al., 2005) in TM2 have been used previously to determine the stoichiometry of other nAChRs. We concluded that injection of oocytes with 1:10 $\alpha 4/\beta 2$ cDNA ratios favors expression of $(\alpha 4)_2(\beta 2)_3$ receptors, whereas 10:1 ratios produce $(\alpha 4)_3(\beta 2)_2$ receptors. We characterized the pharmacological properties of the $\alpha 4\beta 2$ nAChRs with high and low ACh sensitivity and found remarkable differences between them. By exploiting the different pharmacological profile of the receptors, we evaluated the effects of long-term nicotine exposure on the function and receptor numbers of the $\alpha 4\beta 2$ nAChRs with high and low sensitivity to ACh.

Materials and Methods

Materials. *X. laevis* frogs were purchased from Horst Kaehler, (Hamburg, Germany). Acetylcholine (ACh), dihydro- β -erythroidine

(DH β E), (–)-nicotine, *d*-tubocurarine, A-85380, and cytosine were purchased from Sigma-Aldrich (St. Louis, MO). TC-2559 was a gift from Targacept Inc. (Winston-Salem, NC). [3 H]Cytosine (30.4 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). For the synthesis of halogenated derivatives, cytosine was purified from the seeds of the Mexican plant *Sophora secundiflora* using standard methods. Cytosine was halogenated with bromine, iodine monochloride, or chlorine in acetic acid to produce mixtures of 3- and 5-Br-cytosine, 3- and 5-I-cytosine and 3- and 5-Cl-cytosine, respectively. The products of these reactions were separated by column chromatography on silica gel, crystallized to homogeneity, and characterized by 1 H and 13 C NMR and high-resolution electron-impact mass spectrometry. Definitive structure assignments were based on 1 H- 1 H correlation spectroscopy experiments.

Expression Vectors and Site-Directed Mutagenesis. The human $\alpha 4$ and $\beta 2$ nAChR cDNAs were provided by Professor Jon Lindstrom (University of Pennsylvania, Philadelphia, PA) and subcloned into the pcDNA 3.1(hygro-) expression vector (Invitrogen, Ltd., Paisley, UK) for nuclear injection into oocytes. To minimize differences in the transcription of the two cDNAs that may be brought about by untranslated regions flanking the coding regions of the cDNAs (Briggs et al., 2006), subcloning was performed by polymerase chain reaction using primers annealing at the start and end of the translated sequence of the cDNAs. Using these primers, XbaI and NotI restriction sites were introduced at the 5' and 3' ends, respectively, of both subunit cDNAs for subsequent cloning into the linker region of the expression vector.

A hydrophobic to hydrophilic mutation at position 9' in TM2, $\alpha 4$ L283T or $\beta 2$ L274T (Fig. 2A), was introduced in both subunits using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). The mutant subunits are referred to as $\alpha 4^{LT}$ and $\beta 2^{LT}$ hereafter. The full-length sequence of both subcloned wild subunit and mutant subunit cDNAs was verified by DNA sequencing (Department of Biochemistry, University of Oxford, Oxford, UK).

***X. laevis* Oocyte Expression and Electrophysiological Recordings.** Stage V and VI *X. laevis* oocytes were prepared using standard procedures (Chávez-Noriega et al., 1997). Wild-type or mutant human $\alpha 4$ or $\beta 2$ subunit cDNAs, ligated into the pcDNA3.1(hygro-) expression vector, were dissolved in distilled water at a concentration of 1 μ g/ μ l (spectrophotometric and agarose gel electrophoresis determinations). Mixtures of either wild-type or mutated $\alpha 4$ and $\beta 2$ cDNA at 1:10, 10:1, and 1:1 ratios were injected into the nuclei of oocytes in a volume of 18.4 nl/oocyte, using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA). The total amount of cDNA injected per oocyte was kept constant at 2 ng. After injection, oocytes were incubated at 18°C for 2 to 5 days in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES, and 5 mg/l neomycin, pH 7.6. Recordings were performed 3 to 5 days after injection. Coexpression of $\alpha 4^{LT}$ and $\beta 2^{LT}$ cDNAs in oocytes at a 10:1 ratio markedly increased cell death, but survival was increased by transferring the oocytes from normal Barth's medium to a medium supplemented with 100 μ M mecamylamine, an nAChR ion channel blocker, 48 h after injection. The higher incidence of mortality among oocytes expressing LS $\alpha 4^{LT}\beta 2^{LT}$ nAChR seems to be due to an increase in Ca²⁺ entry into the cells brought about by longer spontaneous openings (Revah et al., 1991). Oocytes were placed in a 0.1-ml recording chamber and perfused with modified Ringer's solution (150 mM NaCl, 2.8 mM KCl, 10 mM HEPES, and 1.8 mM BaCl₂, pH 7.2, adjusted with NaOH) at a rate of 10 ml/min. We chose a nominally Ca²⁺-free solution to minimize the contribution to the response of Ca²⁺-gated chloride channels which are endogenous to the *X. laevis* oocyte and may be activated by Ca²⁺ entry through the nAChRs.

Oocytes were impaled by two agarose-cushioned microelectrodes filled with 3 M KCl (0.5–2.0 M Ω) and voltage-clamped at –60 mV using a GeneClamp 500B amplifier and pCLAMP 6 software (Molec-

ular Devices, Sunnyvale, CA). In general, traces were filtered at 1 kHz during recording and digitized at 0.5 to 5 kHz using the Digi-Data 1200 interface (Molecular Devices). All experiments were carried out at room temperature. Agonist concentration-response curves were obtained by normalizing agonist-induced responses to the control responses induced by 1 mM ACh (a near-maximal effective concentration at receptors obtained with 10:1 or 1:1 $\alpha 4\beta 2$ cDNA transfecting ratios but an EC_{50} concentration at receptors expressed by oocytes injected with 1:10 $\alpha 4\beta 2$ cDNA ratios). A minimum interval of 4 min was allowed between agonist applications because this was found to be sufficient to ensure reproducible recordings. The sensitivity of the receptors to inhibition by the nAChR antagonists DH β E and *d*-tubocurarine was tested by first superfusing the antagonist for 2 min and then coapplying it with an EC_{50} of ACh (high sensitivity $\alpha 4\beta 2$ nAChR EC_{50} , 4 μ M; low sensitivity $\alpha 4\beta 2$ nAChR EC_{50} , 88 μ M). Antagonist concentration response data were normalized to the appropriate ACh EC_{50} .

Long-Term Nicotine Exposure. The effects of long-term nicotine exposure on the functional expression of high- and low-sensitivity $\alpha 4\beta 2$ nAChRs were examined by determining the effects of ACh, TC-2559, and 5-I-cytisine in oocytes injected with 1:1 $\alpha 4\beta 2$ cDNA ratios, unless otherwise mentioned. The reporter mutation approach was not used to monitor changes in the function of $\alpha 4\beta 2$ nAChRs because the leftward shifts in the ACh concentration-response curves caused by the reporter mutation or by changes in subunit composition would overlap, making it difficult to separate both effects (compare curves in Fig. 2, B and D). Injected oocytes were incubated in 5 μ M nicotine-containing Barth's solution for 72 h (or 96 h for some [3 H]cytisine binding assays) at 18°C. Exposure to nicotine commenced immediately after injection or 36 h after injection. In agreement with previously published studies (Jia et al., 2003; López-Hernández et al., 2004) the ACh responses of oocytes expressing $\alpha 4\beta 2$ nAChRs were depressed by long-term exposure to 5 μ M nicotine. Therefore, before recordings, oocytes were maintained for 4 h in 15 ml of nicotine-free Barth's solution in a Petri dish (one oocyte per Petri dish) placed on a rotating platform. The nicotine-free medium was replaced with fresh medium every 60 min. This washing procedure permitted sufficient recovery from receptor desensitization to enable reproducible and reliable electrophysiological recordings. The ACh responses of control oocytes (cells not exposed to nicotine) were monitored over the same period as those of nicotine-treated oocytes.

Microtransplantation of Membranes Containing Human $\alpha 4\beta 2$ nAChR to Oocytes. Membranes of HEK293 cells stably expressing human $\alpha 4\beta 2$ nAChR (HEK-h $\alpha 4\beta 2$) were injected into the cytoplasm of oocytes using methods reported previously (Miledi et al., 2002; Palma et al., 2003). HEK-h $\alpha 4\beta 2$ cells were grown as described previously (Zwart et al., 2006). Cells (2.4×10^8 cells) were homogenized with 2 ml of a solution containing 200 mM glycine, 150 mM NaCl, 50 mM EGTA, 50 mM EDTA, and 300 mM sucrose, supplemented with protease inhibitors, pH 9 (Palma et al., 2003). The homogenate was first centrifuged for 15 min at 9500g with a TL-100-4 rotor at 4°C in a TL100 centrifuge (Beckman Coulter, Fullerton, CA). The resulting supernatant was centrifuged for 2 h at 100,000g with the same rotor and centrifuge used in the first centrifugation step. The pellet was resuspended in 5 mM glycine to a concentration of 9 to 14 mg of protein/ml. Oocytes were injected with 100 nl of this preparation. Total protein was assayed using the BCA protein assay kit (EMD Biosciences, Inc., Darmstadt, Germany). Injected oocytes were maintained in Barth's medium, and recordings were made 72 h after injection.

[3 H]Cytisine Binding Assays. Batches of 20 oocytes expressing $\alpha 4\beta 2$ receptors were homogenized using a glass homogenizer in 10 ml of a buffer containing 340 mM sucrose, 1 mM EGTA, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor. Homogenates were centrifuged at 4°C at 1000g for 10 min. The supernatant was removed for further centrifugation, avoiding disturbance of both the surface lipid layer and the pellet that contained

pigment granules, both of which increased nonspecific binding. The supernatant was centrifuged for 1 h at 100,000g. The resulting pellet was resuspended in binding saline (20 μ l per oocyte) of the following composition: 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, 1% bovine serum albumin (w/v), pH 7.5, plus 1 mM EGTA, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, supplemented with 10 μ g/ml each aprotinin, leupeptin, and pepstatin A to protect the receptors. [3 H]Cytisine binding assays were performed by adding a maximal concentration of [3 H]cytisine (5 nM) to the resuspended membranes for 75 min at 4°C, and 10 μ M nicotine was used to define nonspecific binding. Bound and free fractions were separated by rapid vacuum filtration through GF/B filters (Whatman, Maidstone, UK) presoaked in binding saline with 0.1% polyethylenimine. Radioactivity was determined by liquid scintillation counting using Ecoscint H (National Diagnostics, Atlanta, GA).

Data Analyses. The concentration of ACh that evokes 50% of maximum response [EC_{50} and 95% confidence interval (CI)] and Hill coefficient (n_H , mean \pm S.E.M.) were determined by nonlinear regression. Concentration-response curves were obtained by normalizing the current responses to varying concentrations of ACh to the maximal response observed to a saturating concentration of ACh (1 mM) in each oocyte. Fits to full concentration-response curves for individual oocytes were made independently using Prism 4 (GraphPad Software, San Diego, CA) and then averaged to compare significant differences between groups. Concentration-response data for wild or mutant $\alpha 4\beta 2$ receptors were fitted using one- and two-component sigmoidal dose-response equations, $Y = I_{\max \text{ Bottom}} + (I_{\max \text{ Top}} - I_{\max \text{ Bottom}})/(1 + 10^{(\log EC_{50} - X) \times n_H})$ and $Y = I_{\max \text{ Bottom}} + [(I_{\max \text{ Top}} - I_{\max \text{ Bottom}}) \times \text{Fraction}]/(1 + 10^{(\log EC_{50} - X) \times n_H}) + [I_{\max \text{ Top}} - I_{\max \text{ Bottom}}] \times (1 - \text{Fraction})/(1 + 10^{(\log EC_{50} - X) \times n_H})$, respectively, where X is the logarithm of the agonist concentration, Y is the peak amplitude of the normalized current response, I_{\max} is the maximal current, and n_H is the Hill slope. An F test determined whether the one- or two-site model best fit the data; the simpler one-component model was preferred unless the extra sum-of-squares F test had a p value less than 0.05.

To infer the stoichiometry of the high and low ACh sensitivity $\alpha 4\beta 2$ receptor subtypes, ACh concentration-response data obtained from mutant receptors were analyzed as described previously by Boorman et al. (2000). In brief, ACh concentration-response data were normalized as described previously and then fitted with one- or two-component sigmoidal equations. Fits were carried out constraining the Hill coefficient to 1 to obtain parallel fit curves, as shown in Fig. 2, B and C. The constrained fits were used to estimate the potency ratio (EC_{50} wild-type receptor/ EC_{50} mutant receptor).

The introduction of a 9' hydrophilic mutation into receptors of the Cys-loop family increases the rate of spontaneous openings (Revah et al., 1991; Chang and Weiss, 1999), which increased with the introduction of progressively higher numbers of mutant subunits (Fig. 2, B and D). The current through the spontaneously open channels (I_{spt}) in oocytes expressing the mutant $\alpha 4\beta 2$ receptors was determined (at a holding potential of -60 mV) using 100 μ M mecamylamine, a nAChR channel blocker. Spontaneous openings in oocytes expressing wild-type $\alpha 4\beta 2$ nAChRs were not detected under our experimental conditions.

Binding and concentration-response data were pooled from at least two different batches of oocytes (EC_{50} and 95% CI). Tests of significance were performed using Student's t test, and p values less than 0.05 were considered significant.

Results

Concentration-response curves for whole-cell peak-current responses to ACh were obtained from oocytes injected with 1:1, 1:10, or 10:1 $\alpha 4\beta 2$ cDNA ratios (Fig. 1, A–E). Normalized ACh concentration-response data from oocytes injected with 1:1 $\alpha 4\beta 2$ cDNA ratios were best fitted to a two-component sigmoidal equation ($p = 0.026$, F test, $n = 10$), indicating the

presence of two types of $\alpha 4\beta 2$ nAChRs that differed in their sensitivity to activation by ACh (Fig. 1A). The estimated EC_{50} (and 95% CI (and $n_H \pm S.E.M.$ values for the two components were: high ACh sensitivity component, 1.0 (0.3–2.8) μM and 1.2 ± 0.1 ; low ACh sensitivity component, 97 (66–142) μM and 1.1 ± 0.3 . The relative proportions of the two components were 0.25 ± 0.07 and 0.75 ± 0.09 , respectively. These data are comparable with previously reported data concerning ACh effects on oocytes (Zwart and Vijverberg, 1998; Zwart et al., 2006) or mammalian cells (Buisson and Bertrand, 2001; Nelson et al., 2003) coexpressing heterologously high and low ACh sensitivity $\alpha 4\beta 2$ nAChRs. ACh concentration-response data from oocytes injected with 1:10 or 10:1 $\alpha 4/\beta 2$ cDNA ratios were best fitted to a one-component sigmoidal equation ($p < 0.0001$, F test, $n = 10$), and the slope of the resulting curves was near unity (Fig. 1C; data summarized in Table 3). $\alpha 4\beta 2$ nAChRs expressed by oocytes injected with the 1:10 $\alpha 4/\beta 2$ cDNA ratio displayed high sen-

sitivity to activation by ACh (EC_{50} 4.5 μM), whereas $\alpha 4\beta 2$ nAChRs from oocytes injected with the 10:1 ratio had lower sensitivity to ACh (EC_{50} 88 μM). Hereafter, the $\alpha 4\beta 2$ nAChRs obtained by injecting oocytes with 1:10 or 10:1 $\alpha 4/\beta 2$ cDNA ratios will be referred to as HS $\alpha 4\beta 2$ nAChRs or LS $\alpha 4\beta 2$ nAChRs, respectively.

The amplitude of the current responses elicited by maximal concentrations of ACh and maximal [3H]cytisine binding were significantly higher at $\alpha 4\beta 2$ nAChRs obtained by injecting oocytes with equal amounts of $\alpha 4$ and $\beta 2$ cDNAs (5.0 ± 0.6 μA and 67 ± 6.4 fmol/oocyte, respectively) than at HS $\alpha 4\beta 2$ nAChRs (0.4 ± 0.1 μA , $p < 0.0001$, Student's t test, $n = 7$; 19 ± 3.2 fmol/oocyte, $p = 0.026$, Student's t test, $n = 3$) or LS $\alpha 4\beta 2$ nAChRs (1.5 ± 0.2 μA , $p < 0.0001$, Student's t test, $n = 7$; and 29 ± 1 fmol/oocyte, $p < 0.0042$, Student's t test, $n = 3$) (Fig. 1, B, D, and E), even though the total amount of cDNA injected using the 1:1; 1:10, or 10:1 ratios was kept constant. One explanation for this finding is that equal or

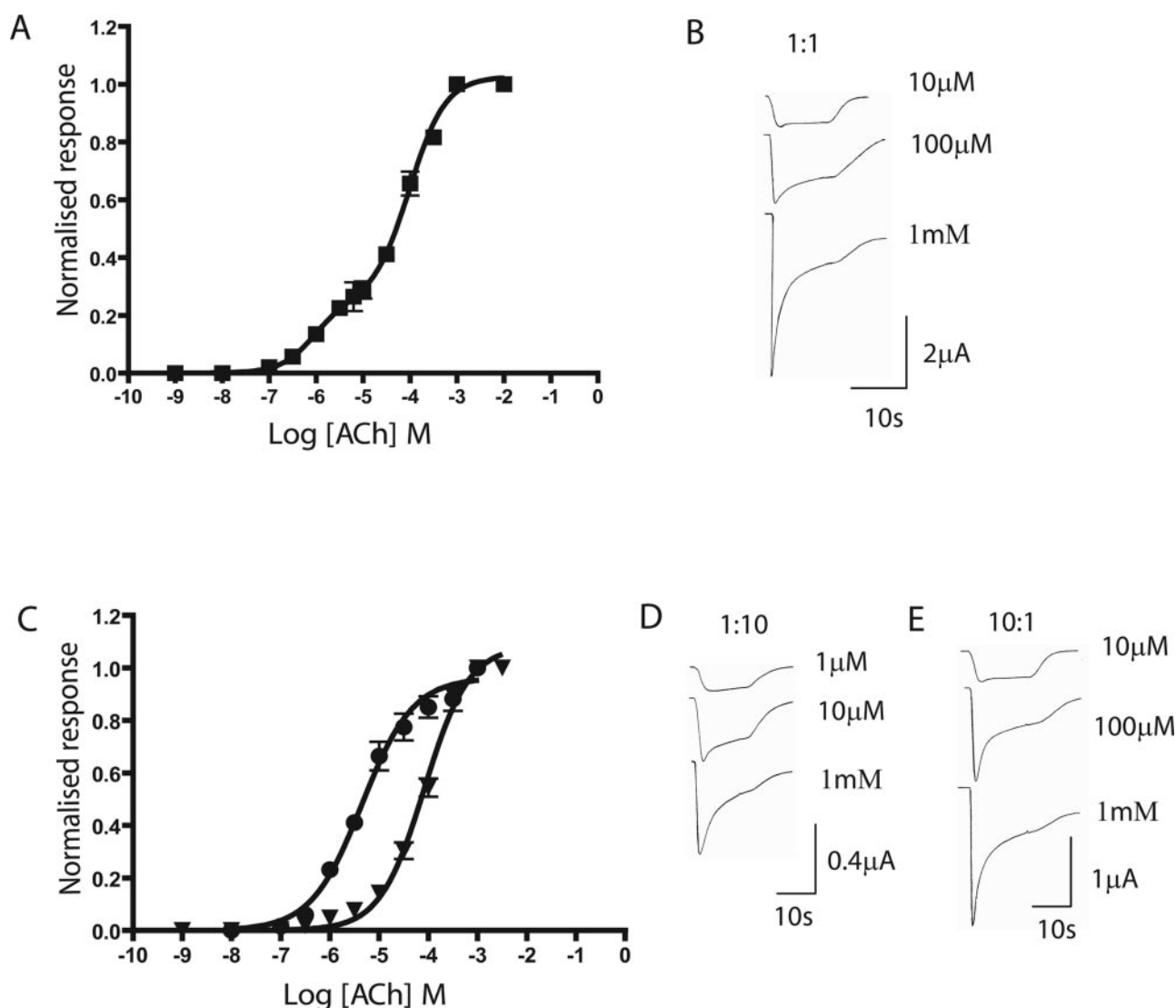


Fig. 1. Effects of $\alpha 4/\beta 2$ cDNA ratios on the sensitivity of human $\alpha 4\beta 2$ nACh to activation by ACh. Concentration-response curves (left) for ACh-induced whole-cell current responses (normalized response relative to that at 1 mM ACh) in oocytes injected with a 1:1 ratio of $\alpha 4/\beta 2$ cDNAs (■, data best fitted to a two-component sigmoidal equation, $p = 0.026$, F test, $n = 10$) (A) or 1:10 (●) or 10:1 (▼) ratios of $\alpha 4/\beta 2$ cDNAs (data best fitted to a one-component sigmoidal equation, $p < 0.0001$, F test, $n = 10$) (C). Parameters from the fits to the data are presented in Table 3. Also shown (right) are representative traces of whole-cell responses under the indicated conditions of $\alpha 4$ and $\beta 2$ cDNA injection ratios (1:1, B; 1:10, D; and 10:1, E) and ACh concentration. Note current amplitude and time calibration bars.

similar levels of $\alpha 4$ and $\beta 2$ subunits may produce higher levels of a critical heteromeric precursor of $\alpha 4\beta 2$ nAChRs (e.g., the tetramer $\alpha 4\beta 2\alpha 4\beta 2$; Kuryatov et al., 2005; Sallette et al., 2005) than extreme subunit ratios such as the 1:10 or 10:1 ratios used in this study, resulting in higher levels of mature receptors. It is noteworthy that the amplitude of current responses evoked by 1 mM ACh (Fig. 1, D and E) ($p = 0.0004$, Student's t test, $n = 7$) and maximal [^3H]cytisine binding (Fig. 6) ($p = 0.0046$, Student's t test, $n = 3$) were significantly smaller in HS $\alpha 4\beta 2$ nAChRs than in LS $\alpha 4\beta 2$ nAChRs. These results, together with the observation that LS $\alpha 4\beta 2$ nAChRs predominate under conditions of equal subunit availability, suggest that LS $\alpha 4\beta 2$ nAChRs may be more efficiently assembled and/or transported to the cell surface than HS $\alpha 4\beta 2$ receptors. In addition, LS $\alpha 4\beta 2$ receptors may have a larger unitary current compared with HS $\alpha 4\beta 2$ receptors. It has been reported previously that high and low agonist sensitivity $\alpha 4\beta 2$ nAChRs display different unitary conductances (Buisson and Bertrand, 2001; Nelson et al., 2003), although these studies had opposite views on which receptor type has the larger unitary conductance.

Stoichiometry of HS and LS $\alpha 4\beta 2$ nAChRs. The preceding results demonstrated that ACh displays monophasic concentration-response curves at HS and LS $\alpha 4\beta 2$ nAChRs. Although we recognize that in principle other types of $\alpha 4\beta 2$ nAChR may have coexpressed with the HS and LS types, we hypothesized that at least one type of receptor was favored for each of the subunit cDNA ratios used in this study. Thus, we used the 1:10 and 10:1 $\alpha 4/\beta 2$ cDNA ratio injection protocol in combination with the L9'T reporter mutation to determine the subunit composition of the HS and LS $\alpha 4\beta 2$ nAChRs heterologously expressed in *X. laevis* oocytes. The subunit stoichiometry of the HS and LS $\alpha 4\beta 2$ nAChRs was determined by analysis of the shifts in the ACh EC_{50} values brought about by the incorporation of mutant subunits into the receptors. Previous studies have shown that the main determinant for the effect of the L9'T mutation on the agonist sensitivity of muscle (Labarca et al., 1995) and $\alpha 7$ - (Palma et al., 1999) and $\alpha 3$ -containing (Boorman et al., 2000) nAChRs is the number of its copies in the receptors. Figure 2, B and D, shows the concentration-response curves to ACh when either $\alpha 4^{\text{LT}}$ or $\beta 2^{\text{LT}}$ were expressed with the wild-type or mutant subunit partner using the 10:1 or 1:10 subunit cDNA ratios, respectively. The ACh concentration-response data for all receptors tested were fitted to a one-component sigmoidal equation, with or without a constrained slope ($p < 0.001 - p < 0.0001$, F test, $n = 4 - 6$), which further supports our view that the 1:10 and 10:1 ratios lead to the expression of homogeneous populations of $\alpha 4\beta 2$ nAChRs. HS or LS $\alpha 4\beta 2$ mutant receptors displayed increased sensitivity to ACh compared with their respective wild-type counterparts as shown by a leftward shift in the ACh concentration-response curves and decrease in the EC_{50} values (Fig. 2, B and D, data summarized in Tables 1 and 2).

For LS $\alpha 4\beta 2$ nAChRs, when both subunits carried the mutation, the ACh sensitivity increased 16,800-fold. When $\alpha 4$ carried the mutation, the ACh sensitivity increased 456-fold, whereas when $\beta 2$ carried the mutation, the increase was only 60-fold. These results show that at the 9' position, both subunits contribute in a multiplicative and symmetric manner to the shifts in ACh EC_{50} values; therefore, the LS $\alpha 4\beta 2$ nAChRs contain more $\alpha 4$ than $\beta 2$ subunits. As reasoned by

Boorman et al. (2000) to infer the stoichiometry of $\alpha 3$ -containing nAChRs, if the stoichiometry of the LS $\alpha 4\beta 2$ nAChR is $(\alpha 4)_3(\beta 2)_2$, then the $\sqrt[3]{}$ of the potency ratio obtained when the mutation is carried only by the $\alpha 4$ subunit (i.e., $\sqrt[3]{456}$), should be equal to the $\sqrt[2]{}$ of the potency ratio obtained when the mutation is carried by the $\beta 2$ subunit (i.e., $\sqrt[2]{60}$). Moreover, the $\sqrt[5]{}$ of the potency ratio obtained when both subunits carry the L9'T mutation should also be similar to the $\sqrt[3]{}$ and $\sqrt[2]{}$ values. As shown in Table 1, the estimated values for $\sqrt[3]{}$, $\sqrt[2]{}$, and $\sqrt[5]{}$ are in agreement with this prediction.

For HS $\alpha 4\beta 2$ nAChRs, there was a 4.2-fold decrease in the value of ACh EC_{50} when the mutation was carried by the $\alpha 4$ subunit (Table 2) whereas when the $\beta 2$ subunit carried the mutation there was an 18-fold decrease in the ACh EC_{50} value. When both subunits carried the L9'T mutation, the ACh EC_{50} value decreased 137-fold. From these results we concluded that HS $\alpha 4\beta 2$ receptors contain more $\beta 2$ than $\alpha 4$ subunits. If the stoichiometry of HS $\alpha 4\beta 2$ nAChRs is $(\alpha 4)_2(\beta 2)_3$, then the $\sqrt[2]{}$ of the potency ratio for HS $\alpha 4^{\text{LT}}\beta 2$ nAChRs should be approximately the same as the $\sqrt[3]{}$ of the potency ratio for HS $\alpha 4\beta 2^{\text{LT}}$ nAChRs, which should also be similar to the $\sqrt[5]{}$ of the potency ratio for HS $\alpha 4^{\text{LT}}\beta 2^{\text{LT}}$ nAChRs. Table 2 shows that the estimated values are in accord with the proposed stoichiometry.

In addition to the increase in ACh sensitivity, oocytes expressing mutant LS or HS $\alpha 4\beta 2$ receptors required a larger holding current to voltage-clamp the membrane at -60 mV compared with oocytes expressing the wild-type receptor. This holding current was blocked by the nAChR ion channel blocker mecamylamine, indicating that it was the result of spontaneously opening mutant $\alpha 4\beta 2$ nAChRs. Figure 2, C and E, shows the relationship between spontaneous openings and type of mutant subunit present in LS or HS $\alpha 4\beta 2$ nAChRs, respectively. The degree of spontaneous openings increased as a function of the number of mutant subunits in the heteropentamers, but the effect was most marked when the receptor was of the LS type. Hydrophilic substitution of the conserved M2 leucine also created spontaneous openly GABA-A receptors (Chang and Weiss, 1999). However, the mutation did not produce spontaneously opening $\alpha 3\beta 4\beta 3$ or $\alpha 3\beta 4$ nAChRs (Boorman et al., 2000). This suggests a difference in the degree of perturbation produced by the mutation in the activation of the two nAChR types.

To test the validity of the proposed subunit combination for the HS and LS $\alpha 4\beta 2$ nAChRs, we plotted the logarithm of the ACh EC_{50} against the presumed number of mutations in each assembled receptor complex. If the observed changes in ACh sensitivity brought about by the L9'T mutation depend only on the number of copies of the mutated subunits in the receptor, the plot should be linear. As shown in Fig. 3, A and C, the relationship between the ACh EC_{50} of the different combinations of LS or HS $\alpha 4\beta 2$ nAChRs and the number of putative L9'T mutations in each receptor type was linear. These results, therefore, are in accord with our conclusion that the stoichiometries of $\alpha 4\beta 2$ nAChRs expressed by *X. laevis* oocytes after injections with 1:10 or 10:1 $\alpha 4/\beta 2$ cDNA ratios are $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$, respectively. Note that the logarithm of fractional spontaneous openings was also linearly related to the number of copies of the mutated subunits in the LS or HS receptors (Fig. 3, B and D).

HS and LS $\alpha 4\beta 2$ nAChRs Display Differential Sensitivities to Nicotinic Agonists. The effects of a range of

nicotinic agonists and antagonists were evaluated in an attempt to pharmacologically differentiate the HS and LS $\alpha 4\beta 2$ nAChRs obtained by 1:10 and 10:1 cDNA injections (Fig. 4;

data summarized in Table 3). Marked differences were observed in the potencies and relative efficacies displayed by nicotinic agonists at HS and LS $\alpha 4\beta 2$ nAChRs. Full concen-

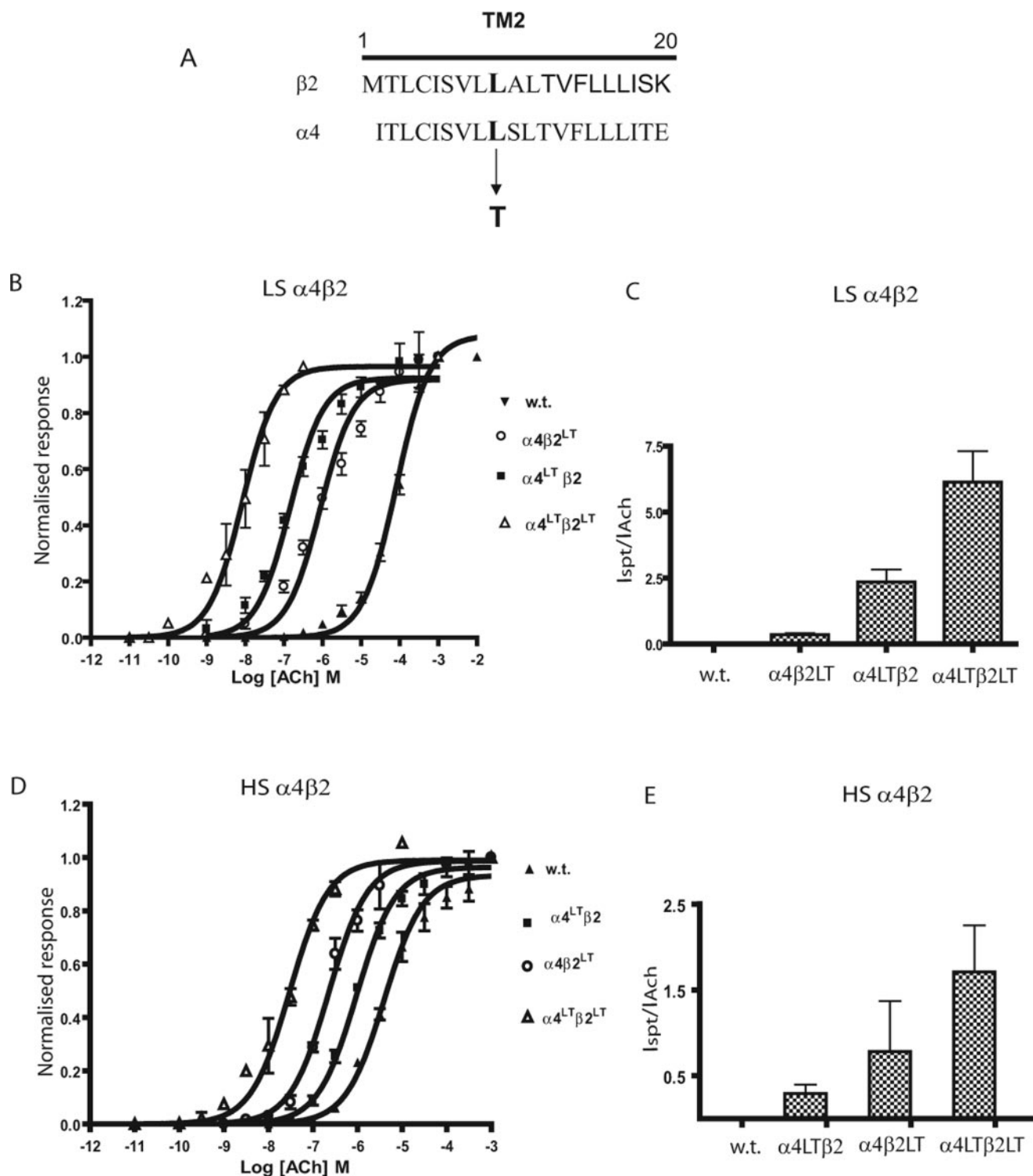


Fig. 2. Effects of the L9'T mutation on the concentration-response curves of ACh at high (HS) and low (LS) ACh sensitivity $\alpha 4\beta 2$ nAChRs. **A**, alignment of the sequences of the second transmembrane domain (TM2) of human $\alpha 4$ and $\beta 2$ nAChR subunits. Leu at position 9' of TM2 was mutated to Thr in both subunits. **B** and **D**, pooled normalized ACh concentration-response data were fitted with the sigmoidal equation under the constraint of equal slopes ($n_H = 1$) to estimate the horizontal distance between the curves as a potency ratio (estimated parameters are shown in Table 1). The data were best fitted to a single sigmoidal equation ($p < 0.001$ – 0.0001 , F test, four to six cells). **C** and **E**, currents through channels spontaneously open (I_{spt}), determined using $100 \mu M$ mecamylamine, and the maximum ACh-activated currents (I_{ACh}) were measured and plotted as a ratio. The recordings were carried out at the holding potential of -60 mV. Note that I_{spt} increased as a function of the number of mutant subunits in the receptors.

Inward currents elicited by EC₅₀ of ACh at either HS or LS $\alpha 4\beta 2$ nAChRs were inhibited by the $\alpha 4\beta 2$ nAChR antagonist DH β E in a concentration-dependent and monophasic manner ($p < 0.0001$, F test, $n = 6$) (Fig. 4B). HS $\alpha 4\beta 2$ receptors were more sensitive to inhibition by DH β E [IC₅₀, 17 (14–19) nM] than LS $\alpha 4\beta 2$ nAChRs [IC₅₀, 410 (361–485) nM]. The current responses of HS or LS $\alpha 4\beta 2$ nAChR to EC₅₀ concen-

Overall, the findings of these studies give additional support to our view that the 1:10 and 10:1 $\alpha 4\beta 2$ subunit cDNA injection ratios produce homogeneous populations of HS and LS $\alpha 4\beta 2$ nAChRs, respectively. Although, in principle, other subunit combinations may form functional receptors [e.g., $\alpha 4(\beta 2)_4$; López-Hernández et al., 2004], in view of the pharmacological differences between the HS and LS $\alpha 4\beta 2$ nAChRs, it is unlikely that other putative subunit combinations would display ligand sensitivities that were so similar to those of the HS and LS $\alpha 4\beta 2$ nAChR types as to render them indistinguishable from them.

Nicotine Up-Regulates HS $\alpha 4\beta 2$ nAChRs in *X. laevis* Oocytes. When mammalian cell lines heterologously expressing a mixture of high and low ACh sensitivity $\alpha 4\beta 2$ nAChRs are exposed to nicotine for a long period of time, there is a selective increase in the function of $\alpha 4\beta 2$ nAChRs with high ACh sensitivity (Buisson and Bertrand, 2001; Nelson et al., 2003; Kuryatov et al., 2005; Sallette et al., 2005; Vallejo et al., 2005). To determine whether human HS $\alpha 4\beta 2$ receptors in *X. laevis* oocytes are up-regulated similarly, oocytes injected with the 1:1 $\alpha 4/\beta 2$ cDNA ratio were exposed to 5 μ M nicotine immediately after injection for a period of 72 h and were washed for 4 h; the responses to ACh, TC-2559, and 5-I-cytisine were then determined. When oocytes injected with equal amounts of $\alpha 4$ and $\beta 2$ cDNAs were exposed to nicotine immediately after injection (i.e., before any intermediate species or mature receptor could have assembled; Kuryatov et al., 2005; Sallette et al., 2005), the expressed $\alpha 4\beta 2$ nAChRs displayed only one EC₅₀ value for ACh. The

EC₅₀ are the means of EC₅₀ estimates obtained by fitting separately each concentration-response curve with a one-component sigmoidal equation, which gave the best fit for all data sets. Potency ratios were calculated from fits in which curves were constrained to slope equal to 1 and are expressed in relation to receptors containing 5 L9' (which therefore have dose ratio values of 1). Values represent data from three to six experiments (*n*).

CI, confidence interval; LS, low sensitivity.

Oocytes were injected with a 1:10 $\alpha 4\beta 2$ cDNA ratio to produce HS $\alpha 4\beta 2$ nAChRs. EC₅₀ values are the means of EC₅₀ estimates obtained by fitting separately each concentration-response curve with a one-component sigmoidal equation, which gave the best fit for all data sets. Potency ratios were calculated from fits in which curves were constrained to slope equal to 1 and are expressed in relation to receptors containing 5 L9' (which therefore have dose ratio values of 1). Results are from four or five independent experiments (*n*).

CI, confidence interval; HS, high sensitivity.

ACh EC_{50} value estimated from the monophasic curve ($p < 0.005$, F test, $n = 5$) was 3 (1.83–7.63) μM , which was not significantly different from the ACh EC_{50} at HS nAChRs expressed by oocytes injected with the 1:10 $\alpha 4/\beta 2$ cDNA ratio. We next evaluated the effects of TC-2559, which is more efficacious than ACh at HS $\alpha 4\beta 2$ nAChRs (but of similar potency at both receptors) and the effects of 5-I-cytisine, which activates only LS $\alpha 4\beta 2$ nAChRs (Table 3). As shown in Fig. 5B, long-term exposure to nicotine increased the maximal response to TC-2559 by 540%. As expected from the negligible difference in the EC_{50} values of TC-2559 at HS and LS $\alpha 4\beta 2$ nAChRs (Table 3), concentration-response curves for TC-2559 at control and nicotine-exposed oocytes were

monophasic ($p < 0.001$, F test, $n = 5$), and the estimated EC_{50} values [control, 2.4 (1.0–5.4) μM ; nicotine-treated, 5.4 (3.4–10) μM] were not significantly different from each other. In contrast, nicotine decreased the relative efficacy of 5-I-cytisine from 50 to 0.94% (Fig. 5C). These results show that in response to nicotine, oocytes favored expression of HS $\alpha 4\beta 2$ nAChRs, even though they were injected with $\alpha 4/\beta 2$ cDNA ratios that produced a mixture of $\alpha 4\beta 2$ nAChRs enriched in LS receptors in oocytes not exposed to nicotine.

We next evaluated how oocytes expressing a mixture of $\alpha 4\beta 2$ nAChRs dominated by the LS receptor type modify, in response to nicotine, the expression of HS and LS $\alpha 4\beta 2$ receptors. For these experiments, oocytes injected with 1:1

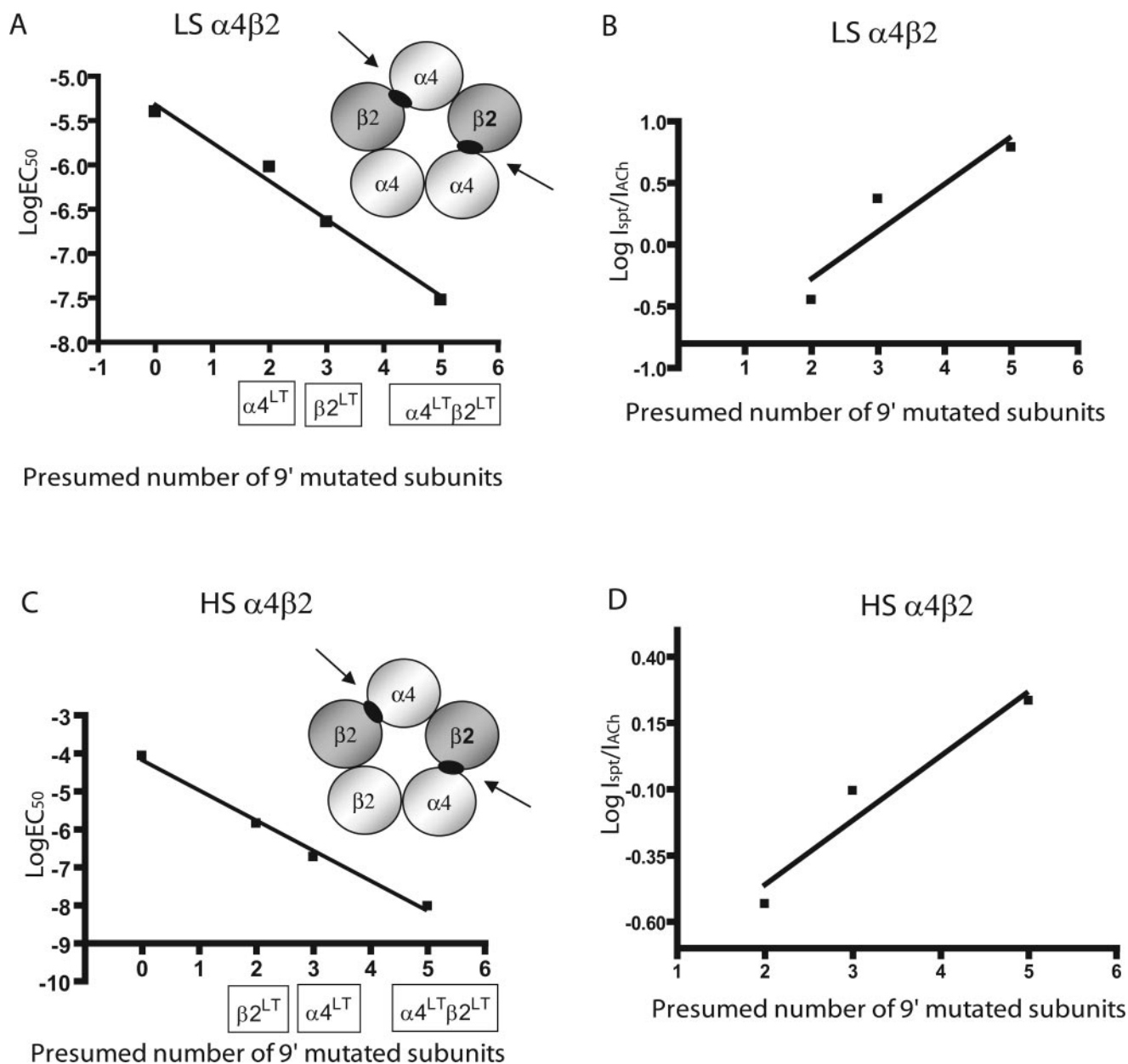


Fig. 3. Relation between the putative number of L9'T mutations in $\alpha 4\beta 2$ nAChRs and the ACh EC_{50} and currents through mutant $\alpha 4\beta 2$ mAChRs spontaneously open. A and C, ACh EC_{50} of $\alpha 4\beta 2$ nAChRs and their putative number of L9'T mutations. ACh EC_{50} values were plotted on a log scale against the number of mutant subunits in either high-sensitivity (HS) or low-sensitivity (LS) $\alpha 4\beta 2$ nAChRs. Note the approximate linearity of the decline in EC_{50} for both types of receptor. The graphs suggest that the $\alpha 4$ subunit is represented three times in the LS receptor and twice in the HS receptor, whereas the $\beta 2$ subunit is represented twice in the LS receptor and three times in the HS receptor. Insets show the subunit arrangement for each $\alpha 4\beta 2$ nAChR subtype. Arrows indicate the agonist binding sites located at $\alpha \beta$ interfaces. B and D, plot of the relationship between the number of mutant subunits in the $\alpha 4\beta 2$ nAChRs and $\log I_{\text{spt}}/I_{\text{ACh}}$.

$\alpha 4\beta 2$ cDNA ratios were exposed to nicotine 36 h after injection, when HS and LS receptor function was clearly detected as shown by the biphasic ACh concentration-response curves obtained at 36 h after injection (Fig. 5D). Oocytes were exposed to nicotine for 72 h. As shown in Fig. 5D, exposure to nicotine 36 h after injection increased the fraction of nAChRs with higher sensitivity to ACh from 0.25 ± 0.07 to 0.49 ± 0.1 of the total in oocytes. The EC_{50} values for the HS and LS components were $1 (0.13-7) \mu M$ and $80 (21-136) \mu M$, which were comparable with those obtained from control oocytes. These results further support the proposal that in response

to nicotine incubation, cells specifically up-regulate $\alpha 4\beta 2$ nAChRs with high ACh sensitivity (Buisson and Bertrand, 2001; Nelson et al., 2003; Kuryatov et al., 2005; Sallette et al., 2005; Vallejo et al., 2005). We also evaluated the effects of TC-2559 and 5-I-cytisine. As shown in Fig. 5E, TC-2559 efficacy increased significantly by 44% [control oocytes, 0.50 ± 0.06 ; nicotine-incubated oocytes, 0.72 ± 0.009 ($p = 0.026$, Student's t test, $n = 4$)], but the efficacy of 5-I-cytisine at nicotine-incubated oocytes (0.44 ± 0.09 ; $n = 4$) was not significantly different from that of control oocytes (0.42 ± 0.04 ; $n = 3$) (Fig. 5E). Thus, our results suggest that nicotine

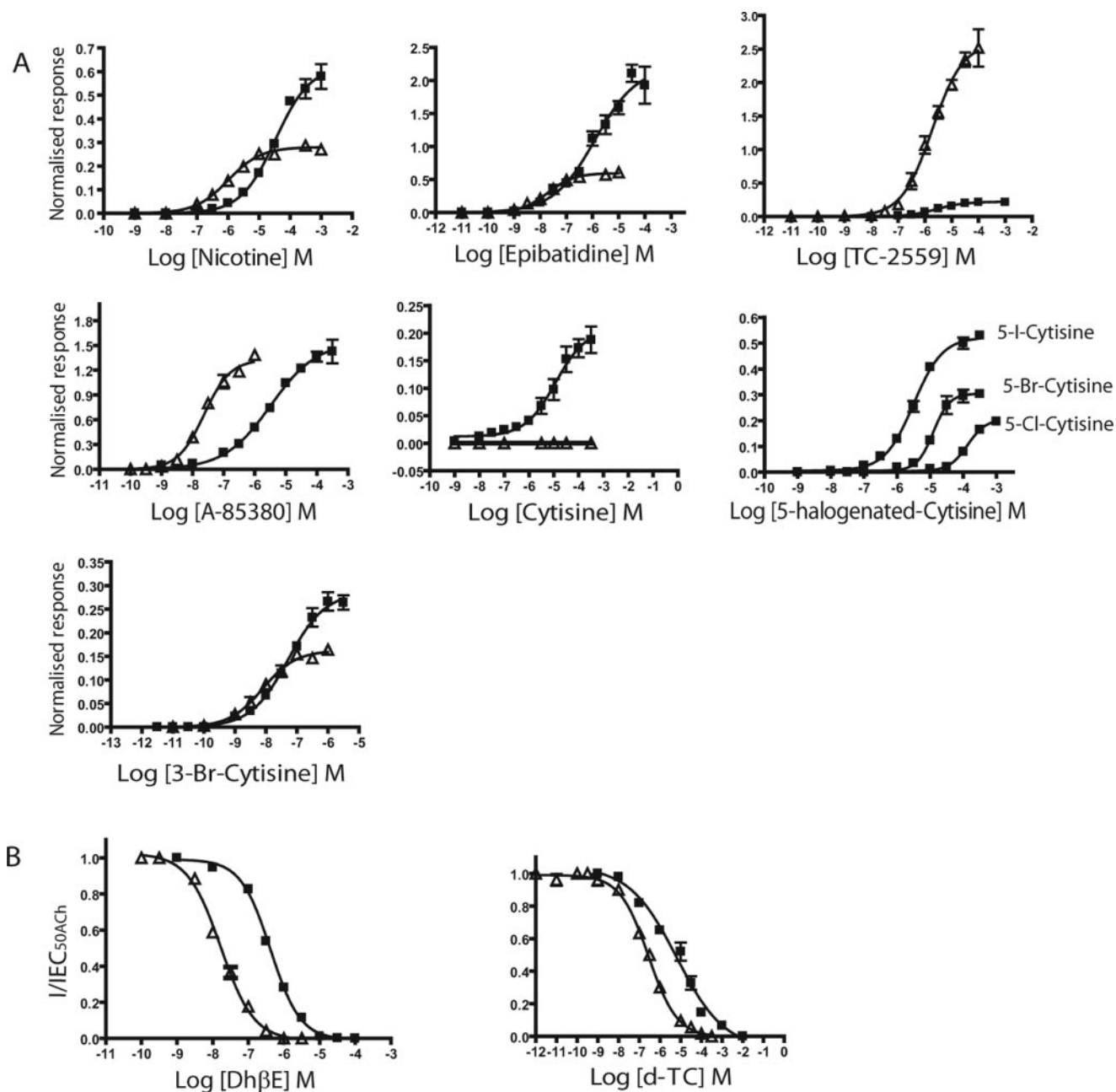


Fig. 4. Concentration-response curves of nicotinic agonists and antagonists on the function of $\alpha 4\beta 2$ nAChR types. Nicotinic agonists (A) and antagonists (B) had different effects at $\alpha 4\beta 2$ nAChRs with high (HS) and low (LS) ACh sensitivity. A, concentration-response curves of nicotinic agonists on the function of HS (Δ) and LS (\blacksquare) $\alpha 4\beta 2$ nAChRs. Note that all agonists tested activated HS and LS $\alpha 4\beta 2$ nAChRs with the exception of 5-halocytisines, which activated current responses at LS $\alpha 4\beta 2$ nAChRs only. Estimated EC_{50} and Hill coefficients for agonists are provided in Table 3. B, concentration-response curves of nicotinic antagonists on EC_{50} ACh responses of HS (Δ) and LS (\blacksquare) $\alpha 4\beta 2$ nAChRs. Estimated IC_{50} for antagonists are provided in text. Agonist or antagonist data were analyzed as described under *Materials and Methods* using a monophasic or biphasic sigmoidal equation. Data points represent the mean of 5 to 10 experiments carried out using at least two different batches of oocytes.

The preceding results generally support the view that nicotine selectively up-regulates HS $\alpha 4\beta 2$ nAChRs (Buisson and Bertrand, 2001; Nashmi et al., 2003; Nelson et al., 2003; Kuryatov et al., 2005; Sallette et al., 2005; Vallejo et al., 2005). It has been suggested that nicotine up-regulates nAChRs by enhancing receptor assembly (Wang et al., 1998; Nashmi et al., 2003; Nelson et al., 2003; Kuryatov et al., 2005; Sallette et al., 2005). An alternative view is that long-term exposure to nicotine alters the functional state of $\alpha 4\beta 2$ nAChR present in the plasma membrane (Buisson and Bertrand, 2001; Vallejo et al., 2005). A straightforward approach to further test these views in *X. laevis* oocytes would be to examine the effects of long-term exposure to nicotine on $\alpha 4\beta 2$ nAChR assembled in HEK-h $\alpha 4\beta 2$ cells and then microtransplanted into *X. laevis* oocytes (Palma et al., 2003). Microtransplanted human $\alpha 4\beta 2$ nAChRs have been shown to display identical pharmacological and functional properties as their in situ counterparts (Palma et al., 2003), which supports their use as a model of mature $\alpha 4\beta 2$ nAChRs. If nicotine up-regulates $\alpha 4\beta 2$ nAChRs by increasing assembly of receptors, then the functional state of microtransplanted $\alpha 4\beta 2$ nAChRs should not be affected by long-term exposure to nicotine. But, if nicotine alters the functional state of $\alpha 4\beta 2$ nAChRs, then microtransplanted receptors exposed to nicotine in oocytes should have altered sensitivity to functional activation. Figure 7A shows that human $\alpha 4\beta 2$ nAChRs isolated from HEK-h $\alpha 4\beta 2$ cells and then microtransplanted into oocytes were of low and high ACh sensitivity types. Concentration-response curves for ACh were best fit to the two-component sigmoidal equation ($p < 0.001$, F test, $n = 6$). Calculations yielded the following EC_{50} values. $\alpha 4\beta 2$ nAChRs with high ACh sensitivity $EC_{50} = 1.6$ (0.5–7.4) μM . $\alpha 4\beta 2$ nAChRs with low ACh sensitivity $EC_{50} = 199$ (158–253) μM . The lower sensitivity component comprised 78% of the overall receptor population. The microtransplanted receptors, therefore, had properties similar to those of the human $\alpha 4\beta 2$ nAChRs expressed by our HEK293 cell line (Zuo et al., 2003) and other HEK293 cell lines (Buisson and Bertrand, 2001; Nelson et al., 2003; Kuryatov et al., 2005) stably expressing human $\alpha 4\beta 2$ nAChRs. We then examined the sensitivity to activation of the microtransplanted receptors after 72 h exposure to nicotine. Figure 7B shows that the concentration-response curve for ACh at microtransplanted $\alpha 4\beta 2$ nAChRs exposed to nicotine was not significantly dif-

We performed additional experiments to assess whether nicotine affects the functional expression of $\alpha 4\beta 2$ nAChRs obtained by injecting oocytes with 1:10 or 10:1 $\alpha 4/\beta 2$ cDNA ratios. We were surprised to find that, in contrast to the $\alpha 4\beta 2$ nAChRs expressed by oocytes injected with 1:1 $\alpha 4/\beta 2$ cDNA ratios, exposure to nicotine did not lead to a significant change in the relative efficacy of 5-I-cytisine or TC-2559 at LS or HS $\alpha 4\beta 2$ nAChRs produced using injections with 10:1 or 1:10 subunit cDNA ratios (data not shown). One explanation for these results could be that the 1:10 or 10:1 cDNA ratios are too extreme for nicotine to exert measurable changes in the functional expression of HS or LS $\alpha 4\beta 2$ nAChRs. Because nicotine-induced nAChR up-regulation is faster in cells with higher levels of receptor expression (Nashmi et al., 2003), up-regulation under extreme subunit ratios, which leads to low receptor expression levels, may be slower than under conditions of balanced subunit abundance. In this case, the effects of nicotine should become detectable with longer periods of incubation. To test this hypothesis, we evaluated binding of 5 nM [3 H]cytisine to membrane homogenates prepared from oocytes that were incubated in nicotine for 72 h or 96 h after injection with 1:1, 1:10, or 10:1 subunit cDNA ratios. As shown in Fig. 6A, nicotine increased binding of 5 nM [3 H]cytisine to membranes from oocytes injected with 1:10 or 10:1 $\alpha 4/\beta 2$ cDNA ratios by less than 1%. In contrast, [3 H]cytisine binding sites in membrane homogenates from oocytes injected with equal amounts of $\alpha 4$ and $\beta 2$ subunit cDNAs were up-regulated from 65 ± 5 fmol/oocyte to 85 ± 3 fmol/oocyte. Incubation in nicotine for 96 h further increased [3 H]cytisine binding to oocytes injected with 1:1 subunit cDNA ratios to 125 ± 7 fmol/oocyte (Fig. 6B). Maximal [3 H]cytisine binding to membranes from oocytes injected with 1:10 cDNA ratios was also increased after 96-h incubation in nicotine (control oocytes, 19 ± 4 fmol/oocyte; nicotine-incubated oocytes, 28 ± 3 fmol/oocyte) (Fig. 6B). Thus, longer periods of incubation were required to up-regulate HS $\alpha 4\beta 2$ nAChRs expressed under conditions of subunit imbalance. It is noteworthy that maximal [3 H]cytisine binding to membrane homogenates prepared from oocytes injected with 10:1 $\alpha 4/\beta 2$ cDNA ratios was not affected by 96-h nicotine incubation (29 ± 5 fmol/oocyte compared with 32 ± 7 fmol/oocyte in control oocytes) (Fig. 6B).

Concentration-response data were obtained and fitted to one- or two-component sigmoidal equations as described under *Materials and Methods*. Data were obtained from at least five oocytes from two different donors. I_{\max} and n_H values are presented as mean \pm S.E.M.

	HS $\alpha 4\beta 2$ nAChR			LS $\alpha 4\beta 2$ nAChR		
	EC ₅₀ (95% CI)	<i>III</i> _{maxACh}	<i>n</i> _H	EC ₅₀ (95% CI)	<i>III</i> _{maxACh}	<i>n</i> _H
	μM					
Ach	4 (3.6–5.7)	1 ± 0.004	0.84 ± 0.02	88 (71–109)	1.1 ± 0.005	0.94 ± 0.01
Nicotine	1 (0.8–1.3)	0.28 ± 0.01	0.78 ± 0.07	34 (23–50)	0.62 ± 0.03	0.83 ± 0.07
Epibatidine	0.02 (0.01–0.03)	0.6 ± 0.02	0.86 ± 0.09	1.3 (0.4–4)	2.2 ± 0.2	0.54 ± 0.1
TC-2559	2 (1–3)	2.6 ± 0.2	0.76 ± 0.2	2.7 (1.2–6.1)	0.22 ± 0.02	0.83 ± 0.2
A-85380	0.03 (0.02–0.04)	1.3 ± 0.06	1 ± 0.1	3.3 (2–5)	1.6 ± 0.06	0.58 ± 0.05
Cytisine	N.D.	N.E.		12 (4–34)	0.22 ± 0.03	0.74 ± 0.04
3-Br-Cytisine	0.008 (0.005–0.01)	0.2 ± 0.009	0.8 ± 0.1	0.05 (0.04–0.08)	0.29 ± 0.01	0.73 ± 0.04
5-I-Cytisine	N.D.	N.E.		3.2 (2–4)	0.53 ± 0.01	0.99 ± 0.08
5-Br-Cytisine	N.D.	N.E.		13 (10–16)	0.31 ± 0.01	1.7 ± 0.4
5-Cl-Cytisine	N.D.	N.E.		14 (94–192)	0.21 ± 0.01	1.50 ± 0.3

N.D., not determined; N.E., no effect detected at highest concentration tested (1 mM).

ferent from that of microtransplanted receptors that were not exposed to nicotine treatment. The ACh concentration-response curves of receptors exposed to nicotine were best fitted to a two-component sigmoidal equation ($p < 0.001$, F test, $n = 6$). The estimated EC_{50} values were: high ACh sensitivity $EC_{50} = 1$ (0.3–1.2) μM ; low ACh sensitivity $EC_{50} = 197$ (154–251) μM . Furthermore, the relative efficacies of TC-2559 or 5-I-cytisine were not changed by nicotine (Fig. 7C). These results are in accord with the first prediction.

Discussion

We report here that injection of *X. laevis* oocytes with human $\alpha 4$ and $\beta 2$ nAChR cDNAs at 1:10 or a 10:1 ratios produced homogeneous populations of $\alpha 4\beta 2$ nAChRs with high or low sensitivity to activation by ACh, respectively. By taking advantage of a hydrophobic-to-hydrophilic mutation

in the 9' position of the TM2 of nAChRs that increases ACh sensitivity, we were able to determine that the stoichiometries of HS and LS receptors are $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$, respectively. Our conclusion is based on the observation that the effects of the mutant subunits on the shift in EC_{50} (as well as on I_{spt}) were symmetrical and multiplicative. Boorman et al. (2000) used the same strategy to infer the stoichiometry of human $\alpha 3\beta 4$ and $\alpha 3\beta 4\beta 3$ receptors expressed heterologously in *X. laevis* oocytes, although they did not determine the shift in the EC_{50} of the double ($\alpha 3^{LT}\beta 4^{LT}$) or triple ($\alpha 3^{LT}\beta 4^{LT}\beta 3^{VT}$ or $\alpha 3^{LT}\beta 4^{LT}\beta 3^{VL}$) mutants.

There are six possible stoichiometries for $\alpha 4\beta 2$ nAChRs: $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ with two agonist binding sites at the $\alpha\beta$ interfaces (Fig. 3, A and C), two other assemblies with only one $\alpha\beta$ binding site or with an alternative binding site at $\alpha\alpha$, $\beta\beta$, or $\beta\alpha$ interfaces [i.e., $(\alpha 4)_4(\beta 2)$, $(\alpha 4)(\beta 2)_4$], and the

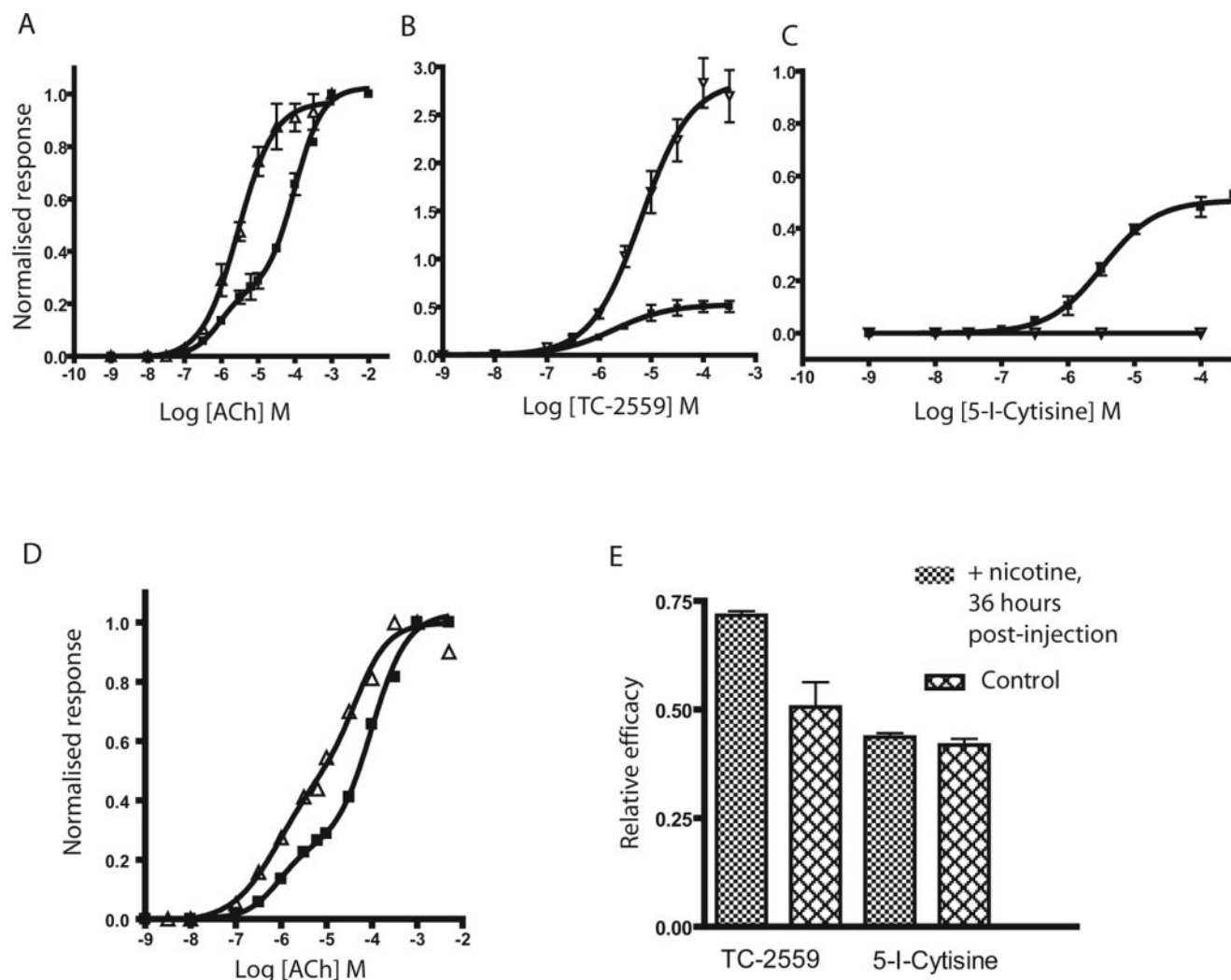


Fig. 5. Effects of long-term nicotine exposure on oocytes injected with a 1:1 $\alpha 4/\beta 2$ subunit cDNA ratio. A, in oocytes injected with 1:1 $\alpha 4/\beta 2$ subunit cDNA, exposed to nicotine immediately and for 72 h subsequently, then studied electrophysiologically, the concentration-response curve of ACh in oocytes was monophasic ($p < 0.005$, F test, $n = 5$) (Δ). In contrast, the effects of ACh in oocytes that were not exposed to nicotine (\blacksquare) were biphasic ($p < 0.0026$, F test, $n = 10$). B, concentration-response curve for TC-2559 effects at oocytes injected with 1:1 $\alpha 4/\beta 2$ cDNA ratio (Δ) and exposed to nicotine immediately after injection and for 72 h subsequently. Control oocytes (\blacksquare) were injected with 1:1 $\alpha 4/\beta 2$ subunit cDNA ratio but were not exposed to nicotine. C, concentration-response curves for 5-I-cytisine effects at oocytes injected with a 1:1 $\alpha 4/\beta 2$ cDNA ratio (Δ) and exposed to nicotine immediately after injection and for 72 h subsequently. Control oocytes (\blacksquare) were injected with the same amount of subunit cDNAs but were not exposed to nicotine. D, exposure to nicotine 36 h after injection with a 1:1 $\alpha 4/\beta 2$ cDNA ratio did not change the biphasic nature of the ACh concentration-response curve, although it increased the high sensitivity component of the biphasic ACh concentration-response curve from 0.25 to 0.49 of the total receptor population. E, bar graph showing that exposure to nicotine 36 h after injection with a 1:1 $\alpha 4/\beta 2$ cDNA ratio increased the efficacy of TC-2559 by 44%, whereas the efficacy of 5-I-cytisine remained unchanged.

stoichiometries $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ with alternative subunit arrangements (Zwart and Vijverberg, 1998). Thus far, however, only the $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ subunit combinations have been detected (Nelson et al., 2003; current study), which suggests that $\alpha 4$ and $\beta 2$ subunits prefer to assemble into those subunit combinations, even under conditions of extreme subunit availability. It may be that only the $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ combinations produce stable receptor complexes, which would explain why only intermediates of approximately the size of the tetramer $\alpha 4\beta 2\alpha 4\beta 2$ have been found so far (Kuryatov et al., 2005; Sallette et al., 2005).

Incorporation of mutant $\alpha 4$ or $\beta 2$ subunits into $\alpha 4\beta 2$ nAChRs caused greater shifts in the potency of ACh and more spontaneous openings at LS than at HS $\alpha 4\beta 2$ nAChRs. This might be caused by the “extra” $\alpha 4$ subunit in the LS receptors, which may give rise to unique molecular properties. The TM2 of the subunits are too similar to contribute to the differences between the HS and LS receptors (Fig. 2A). However, differences in the inner vestibule of both channels may influence the function of the receptors. The vestibules

contain narrow openings made up of residues present in the amphipathic helix (HA-stretch) within the cytoplasmic loop of the subunits of the Cys-loop family (Miyazawa et al., 2003) that influence gating kinetics of nAChRs (Akk and Steinbach, 2000) and single-channel conductance in $\alpha 4\beta 2$ nAChRs and 5HT₃ receptors (Hales et al., 2006). $\alpha 4$ and $\beta 2$ differ in a key residue of the HA-stretch ($\alpha 4$ Phe588 and $\beta 2$ Gln443), which is the residue that is a common determinant of single-channel conductance in 5HT₃ and $\alpha 4\beta 2$ nAChRs (Hales et al., 2006). It is thus conceivable that the extra Phe588 in $(\alpha 4)_3(\beta 2)_2$ or the extra Gln443 in $(\alpha 4)_2(\beta 2)_3$ nAChRs imprint unique molecular properties that manifest most noticeably in the effects of the L9'T mutation in the ACh sensitivity of the LS and HS $\alpha 4\beta 2$ stoichiometries.

Comparisons of the agonist pharmacology of LS and HS $\alpha 4\beta 2$ nAChRs showed intriguing differences, most conspicuously in relative efficacy, which might be useful as leads for the development of $\alpha 4\beta 2$ stoichiometry-selective compounds. Most agonists tested were more potent at HS than LS $\alpha 4\beta 2$ nAChRs and, except for TC-2559 and A-85380, all agonists tested had lower efficacies at HS $\alpha 4\beta 2$ nAChRs. Thus, opening of the pyrrolidine ring of nicotine (TC-2559) or the incorporation of a spacer between the pyridine and pyrrolidine rings (A-85380) of nicotine increases efficacy (and potency) at HS $\alpha 4\beta 2$ receptors but not at LS $\alpha 4\beta 2$ receptors. Cytisine and its 5-halogenated derivatives had markedly higher efficacy at LS $\alpha 4\beta 2$ receptors, which makes them useful tools to study these receptors. Currents were activated at HS $\alpha 4\beta 2$ nAChRs only when the halogen atom was at C3 (3-Br-cytisine). This compound could activate both HS and LS $\alpha 4\beta 2$ nAChRs at nanomolar concentrations, which confirms that halogenation of cytisine at C3 brings about a remarkable increase in potency, which is accompanied by a decrease in receptor subtype selectivity (Cassels et al., 2005, and references therein).

The distinct functional pharmacology of HS and LS $\alpha 4\beta 2$ nAChRs might reflect variations in the environment around the two functional ACh binding sites, which are located at intersubunit contacts between $\alpha 4$ and $\beta 2$ subunits (Corringer et al., 2000). The question of whether both agonist binding sites on the receptor have identical properties has not been addressed for $\alpha 4\beta 2$ nAChRs. In the homologous muscle nAChR, both sites, which occur at $\alpha \delta$ and $\alpha \gamma (\epsilon)$ interfaces, differ in their affinity for nicotinic ligands (Sine and Taylor, 1981). In the case of the $\alpha 4\beta 2$ nAChR, the sites are at $\alpha \beta$ interfaces and are presumed to be identical. However, this may not be so, because of the two binding sites, only one is flanked by the same subunits in both stoichiometries (Fig. 3, A and C). The other site is flanked by $\beta 2$ subunits in the $(\alpha 4)_2(\beta 2)_3$ stoichiometry and by $\alpha 4$ subunits in the $(\alpha 4)_3(\beta 2)_2$ stoichiometry. It is tempting to speculate that the flanking subunits confer subtle differences to the sites that may translate into a different functional pharmacology. On the other hand, residues located at the $\alpha 4$ - $\alpha 4$ interface in $(\alpha 4)_3(\beta 2)_2$ or $\beta 2$ - $\beta 2$ interface in the $(\alpha 4)_2(\beta 2)_3$ combination may form additional binding sites that when bound do not activate the protein but modify the properties of the “classic” ACh binding pocket. At least some of these residues may be homologous to the residues implicated in agonist binding, as is the case for the benzodiazepine allosteric binding site in the GABA_A receptor, which enhances GABA receptor currents (Sigel and Buhr, 1997). We are currently addressing these possibilities.

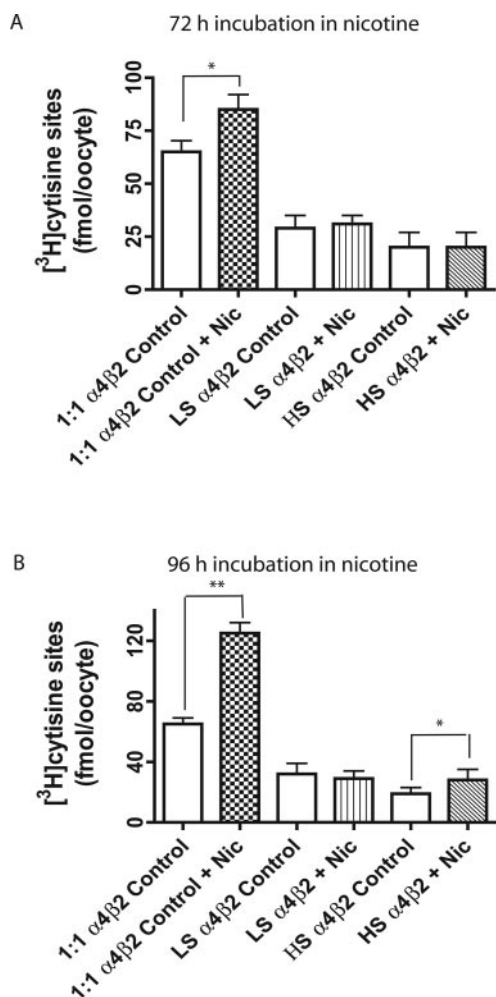


Fig. 6. Effects of long-term exposure to nicotine on maximal binding of [³H]cytisine to membrane homogenates prepared from *X. laevis* oocytes heterologously expressing $\alpha 4\beta 2$ nAChRs. Bar graphs show the effects of 72 h (A) or 96 h (B) exposure to nicotine on maximal binding of [³H]cytisine to oocytes injected with 1:1, 1:10, or 10:1 human $\alpha 4\beta 2$ cDNA ratios. [³H]cytisine binding assays were carried out as described under *Materials and Methods*. *, $p < 0.05$; **, $p < 0.001$, Student's *t* tests.

Two different hypotheses have been proposed to explain nicotine-induced up-regulation of $\alpha 4\beta 2$ nAChRs. One view postulates that nicotine alters the functional state of assembled receptors in the cell surface to a state that binds more [^3H]epibatidine, is more sensitive to activation, and desensitizes more slowly (Buisson and Bertrand, 2001; Vallejo et al., 2005). The other view proposes that nicotine, which is membrane permeant, acts within the endoplasmic reticulum to promote the assembly and maturation of nAChRs (Wang et al., 1998; Nashmi et al., 2003; Kuryatov et al., 2005; Sallette et al., 2005). Our findings generally support the view that nicotine influences assembly and maturation of HS $\alpha 4\beta 2$ nAChRs for the following reasons:

1. Oocytes injected with $\alpha 4/\beta 2$ subunit cDNA ratios that produce a mixture of HS and LS $\alpha 4\beta 2$ nAChRs expressed a homogeneous population of HS $\alpha 4\beta 2$ nAChRs when exposed to nicotine before the initiation of receptor assembly.
2. Nicotine up-regulated HS $\alpha 4\beta 2$ nAChRs in oocytes expressing predominantly LS $\alpha 4\beta 2$ nAChRs without altering the function of LS $\alpha 4\beta 2$ nAChRs. We were surprised to find that maximal [^3H]cytisine binding to oocytes injected

with a 10:1 $\alpha 4/\beta 2$ cDNA ratio was not increased by nicotine, which should have occurred if nicotine enhanced intracellular receptor maturation (Kuryatov et al., 2005; Sallette et al., 2005; Corringer et al., 2006). One explanation for this could be that if a key step in up-regulation of HS $\alpha 4\beta 2$ nAChRs were the interaction of nicotine with an immature species such as $\beta\alpha\beta$ (Corringer et al., 2006), then low levels of $\beta 2$ subunit would slow the process of nicotine-driven maturation of HS $\alpha 4\beta 2$ nAChRs.

3. Exposure to nicotine did not affect the biphasic ACh concentration-response curve of microtransplanted $\alpha 4\beta 2$ nAChRs; i.e., nicotine did not seem to alter the sensitivity to activation of $\alpha 4\beta 2$ nAChRs that were already assembled. We recognize, of course, that our conclusion is tied to the assumption that microtransplantation does not alter the molecular basis of sensitivity to up-regulation by nicotine. Although we have no direct evidence, it seems unlikely that the process of microtransplantation would alter the molecular properties of $\alpha 4\beta 2$ nAChRs so drastically as to render them insensitive to nicotine up-regulation. This view is supported by the finding that neither the ACh sensitivity nor the relative proportions of HS and LS $\alpha 4\beta 2$

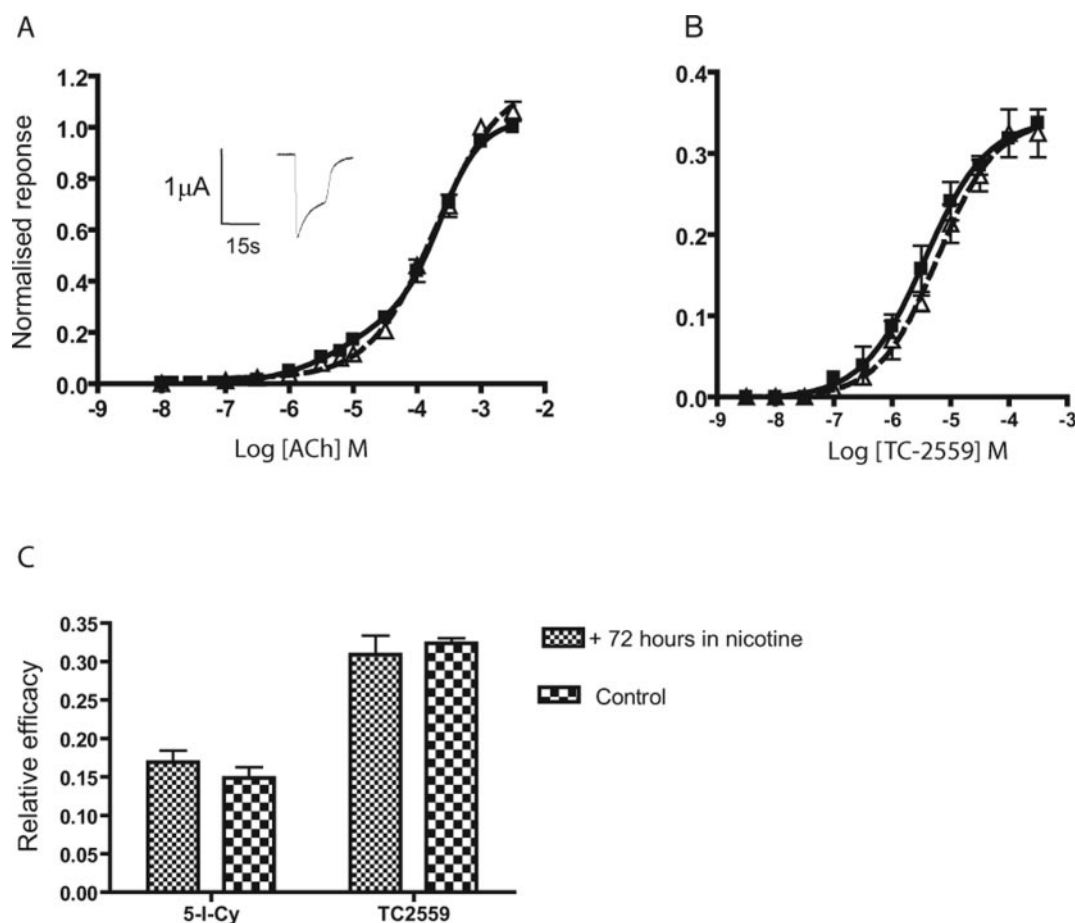


Fig. 7. Effects of long-term nicotine exposure on oocytes expressing microtransplanted human $\alpha 4\beta 2$ nAChRs. The receptors were assembled and expressed first in HEK293 cells stably expressing human $\alpha 4\beta 2$ nAChRs. Oocytes expressing microtransplanted $\alpha 4\beta 2$ nAChRs were incubated in nicotine for 72 h and then used for electrophysiological experiments as described under *Materials and Methods*. A, ACh concentration response data for both control and nicotine-treated oocytes were best fitted to a two-component sigmoidal equation ($p < 0.001$, F test, $n = 6$). Control oocytes, $-\Delta-$; nicotine-incubated oocytes: $-\blacksquare-$. Inset shows a typical current response to 1 mM ACh in HEK293 cell stably expressing human $\alpha 4\beta 2$ nAChRs. B, concentration-response curve for TC-2559 at control ($-\Delta-$) and nicotine-exposed oocytes expressing microtransplanted human $\alpha 4\beta 2$ nAChRs ($-\blacksquare-$). C, bar graph showing the changes in the maximal responses to TC-2559 and 5-I-cytisine in oocytes exposed to nicotine for 72 h and control oocytes. Efficacy values shown represent data normalized to the amplitude of currents elicited by 1 mM ACh.

nAChRs expressed in the HEK293 cells were altered by microtransplantation.

To our knowledge, the question of whether both $\alpha 4\beta 2$ nAChR types occur in neurons has not been resolved. However, it is noteworthy that the functional effects of the $\alpha 4\beta 2$ -preferring agonist A-85380 at HS and LS $\alpha 4\beta 2$ nAChRs are strikingly similar to the effects of this compound in the mouse thalamus, which may coexpress both receptor types (Butt et al., 2002). Moreover, the effects of cytosine, epibatidine and nicotine on HS and LS $\alpha 4\beta 2$ nAChRs receptors were comparable with the effects of these compounds on the "more DH β E-sensitive" and "less DH β E-sensitive" $\beta 2$ -containing receptors that are widely distributed in the mouse brain, suggesting that both stoichiometries may coexist in the brain (Marks et al., 1999). Thus, our approach of expressing homogeneous populations of HS or LS $\alpha 4\beta 2$ nAChRs in combination with the use of stoichiometry-selective $\alpha 4\beta 2$ ligands may be a first step toward the identification of the $\alpha 4\beta 2$ stoichiometries present in the brain and the design of stoichiometry-specific $\alpha 4\beta 2$ nAChRs ligands.

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