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Partial Deletion of the Nicotinic Cholinergic Receptor $\alpha4$ or $\beta2$ Subunit Genes Changes the Acetylcholine Sensitivity of Receptor-Mediated $^{86}Rb^+$ Efflux in Cortex and Thalamus and Alters Relative Expression of $\alpha4$ and $\beta2$ Subunits

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ABSTRACT

$\alpha4$ and $\beta2$ nicotinic cholinergic receptor (nAChR) subunits can assemble in heterologous expression systems as pentameric receptors with different subunit stoichiometries that exhibit differential sensitivity to activation by acetylcholine, yielding biphasic concentration-effect curves. nAChR-mediated $^{86}Rb^+$ efflux in mouse brain synaptosomes also displays biphasic acetylcholine (ACh) concentration-response curves. Both phases are mediated primarily by $\alpha4\beta2^*$-nAChR, because deletion of either the $\alpha4$ or $\beta2$ subunit reduces response at least 90%. A relatively larger decrease in the component of $^{86}Rb^+$ efflux with lower ACh sensitivity occurred with partial deletion of $\alpha4$ ($\alpha4^{+/-}$), whereas a larger decrease in the component with higher ACh sensitivity was elicited by partial deletion of $\beta2$ ($\beta2^{+/-}$). Immunoprecipitation with selective antibodies demonstrated that more than 70% of [^{3}H]epibatidine binding sites in both regions contained only $\alpha4$ and $\beta2$ subunits. Subsequently, $\alpha4$ and $\beta2$ subunit content in the cortex and thalamus of $\alpha4$ and $\beta2$ wild types and heterozygotes was analyzed with Western blots. Partial deletion of $\alpha4$ decreased and partial deletion of $\beta2$ increased the relative proportion of the $\alpha4$ subunit in assembled receptors. Although these methods do not allow exact identification of stoichiometry of the subtypes present in wild-type cortex and thalamus, they do demonstrate that cortical and thalamic nAChRs of the $\alpha4^{+/-}$ and $\beta2^{+/-}$ genotypes differ in relative expression of $\alpha4$ and $\beta2$ subunits a result that corresponds to the relative functional changes observed after partial gene deletion. These results strongly suggest that $\alpha4\beta2$-nAChR with different stoichiometry are expressed in native tissue.

Nicotinic cholinergic receptors (nAChR) are a diverse family of ligand-gated ion channels. Many receptor subunits are expressed in the brain, and they can assemble to form heteropentamers ($\alpha2$, $\alpha3$, $\alpha4$, $\alpha6$, $\beta2$, $\beta4$, $\alpha5$, and $\beta3$) or homopentamers ($\alpha7$) (Lindstrom, 2000). Although many nAChR subtypes have been identified in the brain (Gotti et al., 2006), the most abundant subtype making up binding sites measured with the promiscuous ligand epibatidine (Badio and Daly, 1994; Houghtling et al., 1995; Dávila-García et al., 1997; Marks et al., 1998) is $\alpha4\beta2^*$-nAChR (Zoli et al., 1998; Ross et al., 2000; Marks et al., 2006, 2007). The $\alpha4\beta2^*$-nAChR subtype is also the primary site measured by high-affinity [^{3}H]cytisine or [^{3}H]nicotine binding (Whiting and Lindstrom, 1988; Flores et al., 1992; Picciotto et al., 1995; Marubio et al., 1999).

Because nAChRs are pentameric, the possibility for molecular diversity is substantial. Even within a given subtype, the ratio of subunits assembled in the mature receptor could generate molecular subtypes with distinct physiology and pharmacology. Initial experiments examining heterologously expressed $\alpha4\beta2$-nAChR with high sensitivity to activation by ACh determined a likely stoichiometry of $(\alpha4)_2(\beta2)_3$ (Anand et al., 1991; Cooper et al., 1991). Subsequently, biphasic ACh concentration-effect curves for $\alpha4\beta2$-nAChR have been observed in heterologous expression systems (Zwart and

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; A85380, 3-((2*S*)-azetidinylmethoxy)pyridine; αBgtx, α-bungarotoxin; PMSF, phenylmethylsulfonyl fluoride; Ab, polyclonal antibody; ANOVA, analysis of variance.

Vijverberg, 1998; Covernton and Connolly, 2000; Buisson and Bertrand, 2001; Nelson et al., 2003). Varying the ratio of $\alpha4$ to $\beta2$ altered agonist sensitivity (Zwart and Vijverberg, 1998; Nelson et al., 2003; Khiroug et al., 2004; Moroni et al., 2006). Cells receiving excess $\beta2$ subunits displayed higher sensitivity to ACh ($EC_{50} \approx 1\ \mu M$), and those receiving excess $\alpha4$ subunits displayed lower sensitivity ($EC_{50} \approx 100\ \mu M$). The difference in ACh sensitivity has been attributed to the assembly of $\alpha4\beta2$-nAChR with different α/β stoichiometry. Indeed, injecting mRNA encoding linked $\alpha4\beta2$ subunits with either mRNA encoding $\beta2$ or $\alpha4$ generated ACh concentration effect curves displaying higher or lower ACh sensitivity, respectively (Zhou et al., 2003). These results strongly support the hypothesis that variation in α/β stoichiometry determines relative sensitivity to agonist activation in heterologous expression systems.

Several physiological and biochemical assays for nAChR function in brain preparations have been developed. Included in these is agonist-stimulated $^{86}Rb^+$ efflux (Marks et al., 1999). Biphasic ACh concentration-effect curves for stimulation of $^{86}Rb^+$ efflux from mouse brain synaptosomes, with EC_{50} values comparable with those in the heterologous systems, were observed previously (Marks et al., 1999). Although this assay could be measuring several receptor subtypes, deletion of either $\alpha4$ (Marks et al., 2007) or $\beta2$ (Marks et al., 1999, 2000) eliminated virtually all agonist-stimulated $^{86}Rb^+$ efflux in most brain regions, establishing that most $^{86}Rb^+$ efflux is mediated by $\alpha4\beta2$*-nAChR. The molecular basis for the differential agonist sensitivity observed in native tissues may result from expression of $\alpha4\beta2$*-nAChR that differs in α/β stoichiometry, as is the case with heterologous expression systems. Consequently, nAChR function could be regulated by differences in relative expression of α and β subunits, resulting in altered sensitivity not only in normal activity but also in response to nicotinic drugs (Marks et al., 1999; Briggs et al., 2006; Moroni et al., 2006).

The demonstration that $^{86}Rb^+$ efflux and radiolabeled epibatidine binding in most brain regions is mediated by $\alpha4\beta2$*-nAChR is the basis for the series of studies described here. The approach applied to manipulate gene expression in vivo uses mice that have altered expression of $\alpha4$ and $\beta2$ achieved by the partial gene deletion obtained with heterozygous mutant mice ($\alpha4^{+/-}$ and $\beta2^{+/-}$). Partial or complete deletion of the $\alpha4$ gene has no effect on the expression of $\beta2$ mRNA, and partial or complete deletion of the $\beta2$ gene has no effect on the expression of $\alpha4$ mRNA. However, widespread decrease in the immunoreactivity for both subunits (Whiteaker et al., 2006) as well as a substantial decrease in radiolabeled epibatidine binding (Marks et al., 1999, 2000, 2006, 2007) was observed after partial or complete deletion of either the $\alpha4$ or $\beta2$ gene. Therefore, to examine whether changes in relative expression of $\alpha4$ or $\beta2$ genes affects nAChR expression, we have measured ligand binding and ACh-stimulated $^{86}Rb^+$ efflux in cortical and thalamic synaptosomes. Furthermore, the composition of the assembled receptors was examined by immunoprecipitation using a well characterized panel of subunit specific antibodies (Moretti et al., 2004; Gotti et al., 2005a,b) and by Western blotting using these same antibodies to measure the relative amounts of $\alpha4$ and $\beta2$ subunit protein in assembled receptors in wild-type mice and mice heterozygotic for $\alpha4$ ($\alpha4^{+/-}$) and $\beta2$ ($\beta2^{+/-}$) subunit expression.

Materials and Methods

Materials

(±)-[^{3}H]Epibatine (specific activity, 70.6 Ci/mmol), ^{125}I-A85380 (specific activity, 2200 Ci/mmol), ^{86}RbCl (initial specific activity, 9–18 Ci/mg), and Optiphase Supermix scintillation cocktail were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), and ^{125}I-α-bungarotoxin (αBgtx) (specific activity 220 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Sucrose and HEPES hemisodium salt were obtained from Roche Diagnostics (Indianapolis, IN). Nonradioactive epibatidine, acetylcholine iodide, diisopropylfluorophosphate, PMSF, EDTA, EGTA, bovine serum albumin, atropine sulfate, acetylcholine iodide, tetrodotoxin, Tris, Triton X-100, Tween 20, glucose, nicotine sulfate, NaCl, KCl, $MgSO_4$, $CaCl_2$, and polyethylenimine were purchased from Sigma-Aldrich (St. Louis, MO). CsCl was obtained from Research Products International (Mt. Prospect, IL).

Mice

Animal care and experimental procedures were approved by the University of Colorado Animal Care and Utilization Committee.

Mice engineered by homologous recombination to express null mutation of the $\alpha4$ nAChR subunit (Ross et al., 2000) were originally obtained from the Howard Florey Institute (The University of Melbourne, Melbourne, VIC, Australia). Mice engineered to express the null mutation for the $\beta2$ subunit (Picciotto et al., 1995) were originally obtained from Yale University (New Haven, CT). Mice used in these studies were maintained at the Institute for Behavioral Genetics (University of Colorado, Boulder, CO) in a vivarium maintained at 22 ± 1°C with lights on between 7:00 AM and 7:00 PM. The $\alpha4$ mice had been backcrossed with C57BL/6 mice for two generations, and the $\beta2$ mice had been backcrossed with C57BL/6 mice for 10 generations at the time of experimentation. All animals were produced by mating mice that were heterozygous for the appropriate mutation. Animals were weaned when 25 days old, and they were subsequently housed with like-sexed littermates. All mice were allowed free access to food (Harlan Teklad, Madison, WI) and water.

Genotypes were determined using DNA extracted from tail clippings obtained around 40 days of age as described previously (Salminen et al., 2004). Animals were 60 to 120 days old when used.

Tissue Preparation

Each mouse was killed by cervical dislocation. The brain was removed and placed on an ice-cold platform, and then the cerebral cortex and thalamus were dissected. Tissue samples to be used for functional assays were placed in ice-cold 0.32 M sucrose, buffered to pH 7.5 with 5 mM HEPES hemisodium. Tissue samples to be used for immunochemical studies were rapidly frozen on dry ice.

Crude Synaptosomal Preparation

Cortical and thalamic tissue was homogenized by hand in 10 volumes of the 0.32 M sucrose/HEPES buffer using a glass-Teflon tissue grinder. The subsequent homogenates were centrifuged at 12,000*g* for 20 min. The resulting crude synaptosomal pellets were suspended in isotonic uptake buffer (140 mM NaCl, 1.5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM glucose, and 25 mM HEPES hemisodium salt, pH 7.5). Cortical samples were suspended in 800 μl of buffer and thalamic samples in 350 μl of buffer.

$^{86}Rb^+$ Uptake

Twenty-five-microliter aliquots of synaptosomal suspension were added to 10 μl of uptake buffer containing approximately 4 μCi of $^{86}Rb^+Cl$. Samples were incubated for 30 min at 22°C at which time 5 μl of 80 μM diisopropylfluorophosphate was added to inhibit the acetylcholinesterase, and then samples were incubated an additional 5 min. Uptake was terminated, and synaptosomes were collected by filtration onto A/E glass fiber filters (Gelman Instrument Co., Ann

Arbor, MI) under gentle vacuum (0.8 atmosphere). Filters containing the synaptosomes were then washed with 0.5 ml of uptake buffer.

ACh-Stimulated $^{86}Rb^+$ Efflux

Filters with the synaptosomes loaded with $^{86}Rb^+$ were placed on an open-air platform, and then they were superfused at 22°C with buffer (135 mM NaCl, 1.5 mM KCl, 5 mM CsCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, 1 μM atropine, 50 nM tetrodotoxin, 0.1% bovine serum albumin, and 25 mM HEPES hemisodium, pH 7.5). Buffer was applied to the top of the filter with a Minipuls 3 peristaltic pump (Gilson, Inc., Middleton, WI) at a rate of 2.5 ml/min, and it was removed from the bottom of the filter with a second peristaltic pump set to a faster rate of 3.2 ml/min to actively remove buffer and prevent pooling. The effluent was pumped through a 200-μl Cerenkov cell in a β-Ram HPLC detector (IN/US Systems, Tampa, FL) to monitor radioactivity continuously.

Samples were superfused for 5 min to allow basal efflux to stabilize before beginning data collection. Concentration-effect curves were constructed by exposing each filter to a single concentration of ACh (0.1–1000 μM) for 5 s. A complete concentration-effect curve was constructed for both brain regions from each mouse assayed.

^{125}I-A85380 Binding

Synaptosomes remaining after the measurement of $^{86}Rb^+$ efflux were diluted in 0.1× uptake buffer and centrifuged at 12,000*g* for 15 min. The resulting pellet was suspended in the hypotonic buffer and centrifuged three additional times. The resulting sample was used to measure ^{125}I-A85380 binding (Mukhin et al., 2000). Membranes were incubated at 22°C for 2 h with one of eight concentrations of ^{125}I-A85380 (between 2.0 and 300 pM) in a final volume of 30 μl of uptake buffer minus glucose. Inclusion of 100 μM cytisine established the blank. Binding was terminated by filtration onto GF/B glass fiber filter treated with 0.5% polyethylenimine in a 96-well plate (Unifilter; Whatman, Clifton, NJ) using a cell harvester (Inotech Biosystems, Rockville, MD). Filters were subsequently washed five times with ice-cold buffer. Radioactivity was measured using a Wallac 1450 Microbeta scintillation counter (PerkinElmer Life and Analytical Sciences) after addition of 50 μl of Optiphase Supermix scintillation cocktail to each well of the 96-well counting plate. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Antibody Production and Characterization

The subunit-specific polyclonal antibodies (Abs) used were produced in rabbit against peptides derived from the C-terminal or intracytoplasmic loop regions of rat, human, or mouse subunit sequences, and they were affinity-purified as described previously (Zoli et al., 2002). Most of the Abs have been described previously (Zoli et al., 2002; Champtiaux et al., 2003; Moretti et al., 2004; Gotti et al. 2005a,b).

Antibody specificity was checked by means of quantitative immunoprecipitation or immunopurification experiments using nAChRs from different areas of the central nervous system of wild-type and null mutant mice, which allowed selection of Abs specific for the subunit of interest, and established the immunoprecipitation capacity of each Ab (Zoli et al., 2002; Champtiaux et al., 2003; Moretti et al., 2004). Ab specificity was also tested by Western blotting, which showed that some of the Abs were less specific than in the immunoprecipitation experiments. Frequent monitoring of Ab properties is required because specificity is not only sequence-related (the same peptide can be used to raise Abs with different degrees of specificity in different rabbits) but can also change over time in the same rabbit.

Preparation of Cortex and Thalamus Membranes and 2% Triton X-100 Extracts from α4 or β2 Genotypes

The cortex and thalamus were dissected, immediately frozen on dry ice, and stored at −80°C for later use. In every experiment, the tissues from cortex (0.3–0.35 g) or thalamus (0.1–0.15 g) were homogenized in an excess (10 ml) of buffer (50 mM Na-phosphate, pH 7.4; 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM PMSF) for 2 min in an UltraTurrax homogenizer (Janke and Kunkle, Staufen, Breisgau, Germany). The homogenates were then diluted and centrifuged for 1.5 h at 60,000*g*. The 2% Triton X-100 extracts were prepared as described previously (Gotti et al., 2005a,b). Protein content of the membranes, 2% Triton X-100 extracts, and concentrated gradient fractions (see below) was measured using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

[^{3}H]Epibatidine Binding

To ensure that the α7-containing subtypes did not contribute to [^{3}H]epibatidine binding (Marks et al., 2006), both in membrane as well as in solubilized receptors (present in the extract and immunoprecipitation experiments), the binding were performed in the presence of 2 μM αBgtx, which specifically binds to α7*-nAChR (and thus prevents [^{3}H]epibatidine binding to these sites).

Membrane. Binding to the homogenates obtained from cortical or thalamic membranes was carried out overnight by incubating aliquots of the membrane with 2 nM [^{3}H]epibatidine at 4°C. Nonspecific binding (averaging 5–10% of total binding) was determined in parallel samples containing 100 nM unlabeled epibatidine.

At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine, washed with 15 ml of buffer (10 mM Na-phosphate, pH 7.4, and 50 mM NaCl), and counted in a beta counter.

Solubilized Receptor. The Triton X-100 extracts were labeled with 2 nM [^{3}H]epibatidine. Tissue extract binding was performed using DE52 ion-exchange resin (Whatman, Maidstone, UK) as described previously (Vailati et al., 1999).

Immunoprecipitation of [^{3}H]Epibatidine-Labeled Cortical Receptors by Anti-Subunit-Specific Antibodies

The extracts (100–150 μl) obtained from cortical and thalamic tissues of mice differing in α4 and β2 genotypes were labeled with 2 nM [^{3}H]epibatidine, and then they were incubated overnight with a saturating concentration of anti-subunit affinity-purified IgG (anti-α2, -α3, -α4, -α5, -α6, -β2, -β3, or -β4). Each immunoprecipitate was recovered by incubating the samples with beads containing bound anti-rabbit goat IgG (Technogenetics, Milan, Italy). The level of immunoprecipitation with each Ab was expressed as the percentage of [^{3}H]epibatidine-labeled receptors immunoprecipitated by the Abs (taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%) or as femtomoles of immunoprecipitated receptors per milligram of protein.

α4β2 Subtype Immunopurification and Analysis

The extracts prepared from mouse cortex ($\beta2^{+/+}$) were incubated twice with 5 ml of Sepharose-4B and bound anti-β2 Abs to remove the β2 receptors from the extract. The bound β2*-nAChRs were subsequently eluted from the Sepharose-4B with 0.2 M glycine, pH 2.2, or by competition with 100 μM concentrations of the corresponding β2 peptide used for Ab production. The subunit content of the purified receptors was determined by immunoprecipitation using the purified subtypes eluted with the peptides labeled with 2 nM [^{3}H]epibatidine and the subunit-specific antibodies.

Sucrose Gradient Centrifugation

To analyze the subunit stoichiometry of the nAChR subtypes, we used 5 to 20% sucrose gradients to separate the assembled nAChRs from the unassembled subunits present in the different genotypes.

To this end, linear 5 to 20% sucrose gradients in phosphate-buffered saline plus 1 mM PMSF and 0.1% Triton X-100 were prepared using a Buckler gradient maker (Buckler, Fort Lee, NJ), and they were stored for 4 h at 4°C before use. The volume of each gradient was 12 ml. Then, 500 μl of 2% Triton X-100 extracts ob-

tained from *Torpedo californica* electric organ (0.5–1 g labeled by incubation with 6 nM ^{125}I-αBgtx) and 500 μl of a 2% Triton X-100 extract of either cortex and thalamus prepared from mice differing in α4 or β2 genotype were loaded onto the gradients and centrifuged for 14 h at 40,000 rpm in a Beckman SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions of 0.5 ml were collected from the top of the gradient and directly counted on a gamma counter (for the *T. californica* gradients), or they were added to the affinity-purified anti-α4 or anti-β2 Abs bound to microwells and processed as described previously (Gotti et al., 2005a,b).

Affinity-purified anti-α4 or anti-β2 Abs (10 μg/ml in 50 mM phosphate buffer, pH 7.5) were bound to microwells (Maxi-Sorp; Nalge Nunc International, Rochester, NY) by overnight incubation at 4°C. The following day, the wells were washed to remove excess unbound Abs. Then, 100-μl aliquots of the gradient fractions that had been diluted 1:2 with a buffer (50 mM Tris-HCl, pH 7, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2 mg/ml bovine serum albumin, and 0.05% Tween 20) were added to the wells and incubated overnight at 4°C. After this incubation, the wells were washed and immobilized receptors quantified using 1 nM [^{3}H]epibatidine to label the binding sites. The wells were then washed seven times with ice-cold phosphate-buffered saline containing 0.05% Tween 20, and the bound radioactivity was recovered by incubation with 200 μl of 2 N NaOH for 2 h. The bound radioactivity was then determined by beta counting.

The peak fractions of the gradients for samples of each of the different genotypes corresponding to the sedimentation coefficient of 10 S were pooled and concentrated by centrifugation in an Amicon Centricon filter (Millipore Corporation, Billerica, MA) with a cutoff of 10,000 Da, and the specific activity of these concentrated pools was determined

Immunoblotting

The protein obtained from concentrated gradient fractions was diluted 1:1 (v/v) with Laemmli buffer, and then it underwent SDS-polyacrylamide gel electrophoresis using 9% acrylamide.

For the experiments using constant protein levels, 5 to 10 μg of protein was loaded in each lane. For experiments using a constant number of binding sites, the amount of protein for each lane was adjusted to have a constant number of binding sites per lane.

After SDS-polyacrylamide gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membranes with 0.45-μm pores (Whatman Schleicher and Schuell, Dassel, Germany). The blots were blocked overnight in 5% nonfat milk in Tris-buffered saline, washed in a buffer containing 5% nonfat milk and 0.3% Tween 20 in Tris-buffered saline, incubated for 2 h with the primary antibody (1–2.5 μg/ml), and then incubated with the appropriate peroxidase-conjugated secondary Abs. After another series of washes, peroxidase was detected using a chemiluminescent substrate (Pierce Chemical)

Densitometric Quantification of Western Blot Bands

The signal intensity of the Western blot bands was acquired using an Epson 4500 gel scanner. The developed films were scanned as a Tiff scale in eight-bit gray scale format at setting of 300 dpi. All of the films obtained from the separate experiments of different $\alpha4^{+/+}$, $\alpha4^{+/-}$, and $\alpha4^{-/-}$, $\beta2^{+/+}$, $\beta2^{+/-}$, and $\beta2^{-/-}$ genotypes were acquired in the same way and scanned in parallel with the calibrated optical density step tablet from Stouffer (Mishawaka, IN).

The images were analyzed using National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/). The pixel values of the images were transformed to optical density values by the program using the calibration curve obtained by acquiring the calibrated tablet with the same parameters as those used for the images.

The immunoreactive bands were quantified in four to five separate experiments for the cortex and in three experiments for the thalamus. For the constant protein, the optical density ratio was calculated by taking the optical density of the wild type as 1. The cortical values are the mean ± S.E.M. of four separate experiments for each genotype obtained from four separate sucrose gradient centrifugations; the thalamic values represent three separate experiments obtained from two different gradient centrifugations.

In the constant binding sites, the concentrated gradient fraction samples with the same number of [^{3}H]epibatidine binding sites were loaded in the lanes for wild-type ($\alpha4^{+/+}$ and $\beta2^{+/+}$) and for heterozygotic ($\alpha4^{+/-}$ and $\beta2^{+/-}$) preparations. To compare the signal intensity of the α4 and β2 Ab labeling in wild types and heterozygotes, the optical density ratio of relative expression was expressed as the heterozygote/wild-type ratio.

Data Calculation and Analyses

Primary data from individual stimulations and ACh concentration-effect curves were analyzed using the nonlinear least-squares algorithm in SigmaPlot 2001 (Systat Software, Inc., Point Richmond, CA). ACh-stimulated $^{86}Rb^+$ efflux was calculated as the increase in radioactivity above baseline, and it was normalized among samples and experiments by dividing by the basal efflux. Basal efflux was calculated by fitting the counts before and after the ACh-stimulated peak to a one-component exponential decay [$EB_t = EB_0 \times \exp(-k \times t$], where EB_t is the amount of basal efflux at time t; EB_0 is the this value at $t = 0$, and k is the first-order decay constant. ACh-stimulated efflux was calculated by subtracting basal efflux calculated from the exponential decay curve from the actual data and dividing by the basal efflux.

ACh concentration effect curves were fit to a two-component model: $E = E_hs\times ACh/(K_{hs} + ACh) + E_{ls} \times ACh/(K_{ls} + ACh)$, where E is the total $^{86}Rb^+$ efflux at each concentration of ACh, and E_{hs} and E_{ls} are the maximal $^{86}Rb^+$ efflux rates for the components with higher (K_{hs}) and lower (K_{ls}) sensitivity to stimulation by ACh. Initially, results for cortex and thalamus of mice of each genotype for which there were measurable responses ($\alpha4^{+/+}$, $\alpha4^{+/-}$, $\beta2^{+/+}$, and $\beta2^{+/-}$) were fit individually providing eight separate determinations of K_{hs} and K_{ls}. No significant differences in either K_{hs} (average value 2.5 μM) or K_{ls} (average value 140 μM) were observed between regions or among genotypes. Subsequently, results for each experiment were fit to the two-component model, with values for K_{hs} and K_{ls} fixed at 2.5 and 140 μM, respectively, to obtain estimates of V_{hs} and V_{ls} for every individual mouse.

SPSS (SPSS, Inc. Chicago, IL) was used for the statistical analyses. Results were evaluated using analysis of variance (ANOVA). Initially, a two-way AVOVA [nAChR subunit (α4 or β2) and genotype (+/+, +/−, and −/−)] was used to analyze the effect of deletion of each gene on both V_{hs} and V_{ls} in thalamus and cortex. Subsequently, the effect of each individual deletion was evaluated with one-way ANOVA to compare activity in +/+, +/−, and −/− for α4 and β2 or by t test to compare α4 and β2 mice.

Results

nAChR Function in Cortical and Thalamic Synaptosomes of α4 and β2 Mice

^{125}I-A85380 Binding in Synaptosomal Membranes. ^{125}I-A85380, which has been identified as a β2-selective ligand (Mukhin et al., 2000), was used to measure binding sites in membrane preparations from mouse thalamic and cortical synaptosomes. To determine the effect of sequential deletion of the α4 or β2 subunit on nicotinic binding sites in mouse cortex and thalamus, saturation curves for ^{125}I-A85380 binding to membranes prepared from synaptosomes were constructed (Fig. 1). Deletion of either the α4 or β2 subunit had no significant effect on the apparent K_D value for ^{125}I-A85380 in either brain region, but the average apparent K_D value for cortex (3.7 pM) was slightly lower than that in

thalamus (5.6 pM). No differences in ^{125}I-A85380 binding were detected between the $\alpha4^{+/+}$ and $\beta2^{+/+}$ wild-type mice. A gene dose-dependent decrease in maximal ^{125}I-A85380 binding was observed after deletion of either the $\alpha4$ subunit (cortex: $F_{2,9} = 247.9$, $p < 0.001$; and thalamus: $F_{2,9} = 76.61$, $p < 0.001$) or the $\beta2$ subunit (cortex: $F_{2,9} = 3737.9$, $p < 0.001$; and thalamus: $F_{2,9} = 137.18$, $p < 0.001$). Binding was reduced by approximately half after deletion of a single copy of either gene in both brain regions ($\alpha4$ thalamus, 48.1 ± 3.3%; $\beta2$ thalamus, 44.9 ± 3.9%; $\alpha4$ cortex, 50.0 ± 4.9%; and $\beta2$ cortex, 44.6 ± 0.9%) and by greater than 90% after deletion of either gene ($\alpha4$ thalamus, 93.7 ± 0.9; $\beta2$ thalamus, 97.6 ± 2.1%; $\alpha4$ cortex, 96.1 ± 2.1%; and $\beta2$ cortex, 98.5 ± 2.5%).

Acetylcholine-Stimulated ^{86}Rb$^+$ Efflux. Concentration effect curves for ACh-stimulated ^{86}Rb$^+$ efflux from both cortical (Fig. 2, A and D) and thalamic (Fig. 3, A and D) synaptosomes of wild-type mice are biphasic. EC_{50} values for the higher and lower sensitivity components did not differ significantly between the brain regions, and they were unaffected by genotype (average EC_{50} value for the higher sensitivity component was 2.5 μM and for the lower sensitivity component was 140 μM). The absolute and relative amounts of the ^{86}Rb$^+$ efflux with higher and lower sensitivity to ACh stimulation differed between these regions, and they are discussed in more detail below. Both phases of ACh-stimulated ^{86}Rb$^+$ efflux were virtually eliminated by deletion of either the $\alpha4$ or $\beta2$ subunit in both regions.

Cortex. The effect of partial or complete deletion of either the $\alpha4$ or $\beta2$ nAChR subunit on ACh-stimulated ^{86}Rb$^+$ efflux in cortical synaptosomes is presented in Fig. 2. The concentration-effect curves for the $\alpha4$ and $\beta2$ wild-type mice are both biphasic, with approximately 70% of the response represented by the lower sensitivity component (Fig. 2, A and D). No significant differences in ACh-stimulated release between the $\alpha4^{+/+}$ and $\beta2^{+/+}$ wild-type mice were noted for either the higher sensitivity components ($\alpha4$, 3.03 ± 0.28; $\beta2$, 3.30 ± 0.28; $t_{26} = 0.86$, $p > 0.05$) or the lower sensitivity components ($\alpha4$, 6.87 ± 0.57; $\beta2$, 7.49 ± 0.30; $t_{26} = 0.53$, $p > 0.05$). Deletion of either subunit reduced ACh-stimulated ^{86}Rb$^+$ efflux more than 90% such that the residual response in the null mutants was not significantly different from zero (Fig. 2, C and F). Total ACh-stimulated ^{86}Rb$^+$ efflux in either $\alpha4^{+/-}$

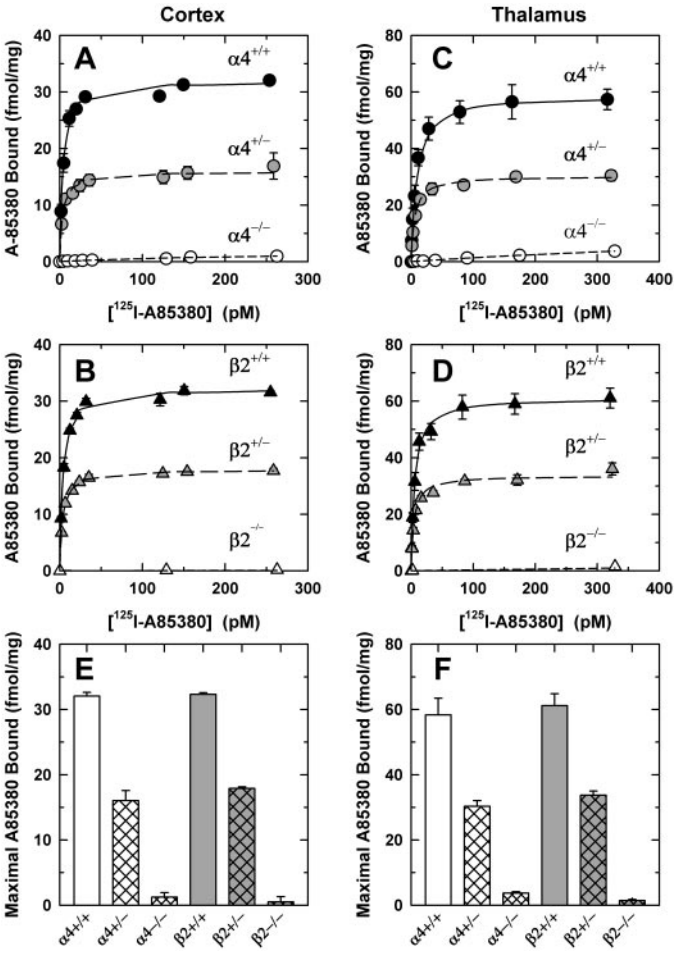

Fig. 1. [^{125}I]A85380 binding in membranes prepared from cortical and thalamic synaptosomes. Saturation curves for ^{125}I-A85380 binding were constructed using membranes prepared from cortical (A, $\alpha4$ mice; B, $\beta2$ mice) or thalamic (C, $\alpha4$ mice; D, $\beta2$ mice) synaptosomes remaining after the measurement of functional responses. The effects of sequential $\alpha4$ and $\beta2$ gene deletion on maximal ^{125}I-A85380 binding in cortex (E) and thalamus (F) are compared. Values represent mean ± S.E.M. of four separate determinations.

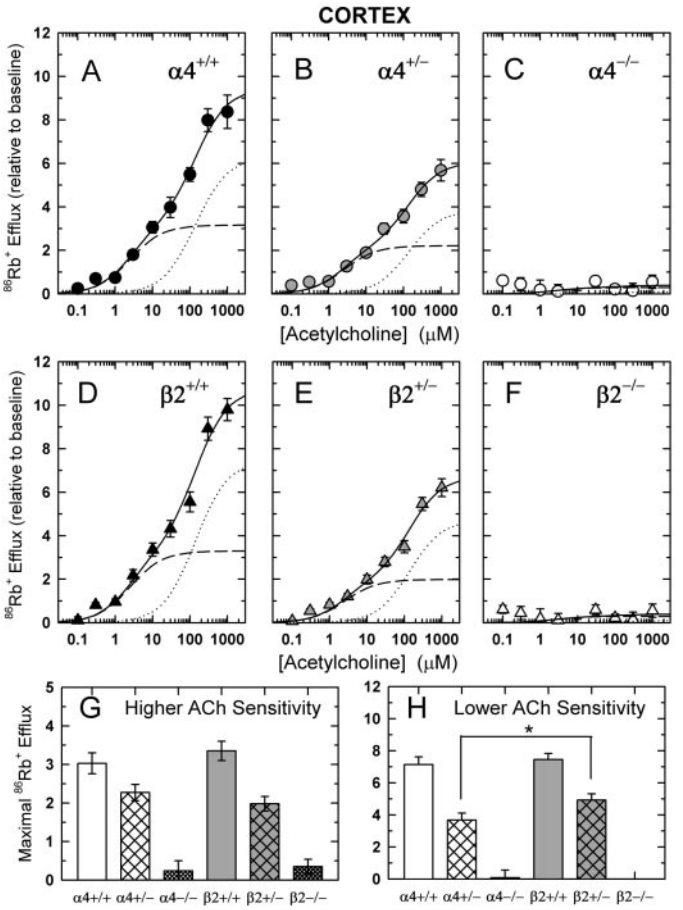

Fig. 2. ACh stimulation of ^{86}Rb$^+$ efflux from cortical synaptosomes. Crude cortical synaptosomes were prepared from cerebral cortex of $\alpha4^{+/+}$ (A; $n = 15$), $\alpha4^{+/-}$ (B; $n = 18$), $\alpha4^{-/-}$ (C; $n = 6$), $\beta2^{+/+}$ (D; $n = 13$), $\beta2^{+/-}$ (E; $n = 16$), and $\beta2^{-/-}$ (F; $n = 4$) mice. ^{86}Rb$^+$ efflux was measured using the indicated [acetylcholine] as described under *Materials and Methods*. Data were fit to a two-component model, and the results of these curve fits are shown with the solid line. Higher and lower sensitivity components are indicated by the dashed and dotted lines, respectively. The effects of $\alpha4$ or $\beta2$ gene deletion on the maximal higher ACh sensitivity and lower ACh sensitivity components of ^{86}Rb$^+$ efflux are shown in G and H, respectively. A significant difference ($p < 0.05$) between $\alpha4^{+/-}$ and $\beta2^{+/-}$ is indicated by the asterisk (*) in H.

(39% decrease) or $\beta2^{+/-}$ (36% decrease) was significantly lower than that in wild-type mice, and the extent of decrease observed for each heterozygote was similar. However, components of ACh-stimulated $^{86}Rb^+$ efflux with higher and lower ACh sensitivity were differentially affected by partial deletion of $\alpha4$ and $\beta2$ (Fig. 2, G and H). A 26 ± 9.6% reduction in the higher sensitivity component was observed for $\alpha4^{+/-}$ mice, whereas a 46 ± 6.9% reduction was observed for this component in $\beta2^{+/-}$ mice (Fig. 2G). A 49 ± 6.9% reduction in the lower sensitivity component was observed for $\alpha4^{+/-}$ mice, whereas a 32 ± 5.7% reduction was observed for $\beta2^{+/-}$ mice. In addition, the residual lower sensitivity $^{86}Rb^+$ efflux in $\beta2^{+/-}$ mice was significantly greater than that in $\alpha4^{+/-}$ mice (4.93 ± 0.38 versus 3.86 ± 0.44; $t_{32} = 2.14$, $p < 0.05$). Thus, in cortical synaptosomes partial deletion of the $\alpha4$ subunit ($\alpha4^{+/-}$) produced a relatively larger decrease in the lower sensitivity component of ACh-stimulated $^{86}Rb^+$ efflux, whereas partial deletion of $\beta2$ ($\beta2^{+/-}$) produced a relatively larger decrease in the higher sensitivity component of ACh-stimulated $^{86}Rb^+$ efflux.

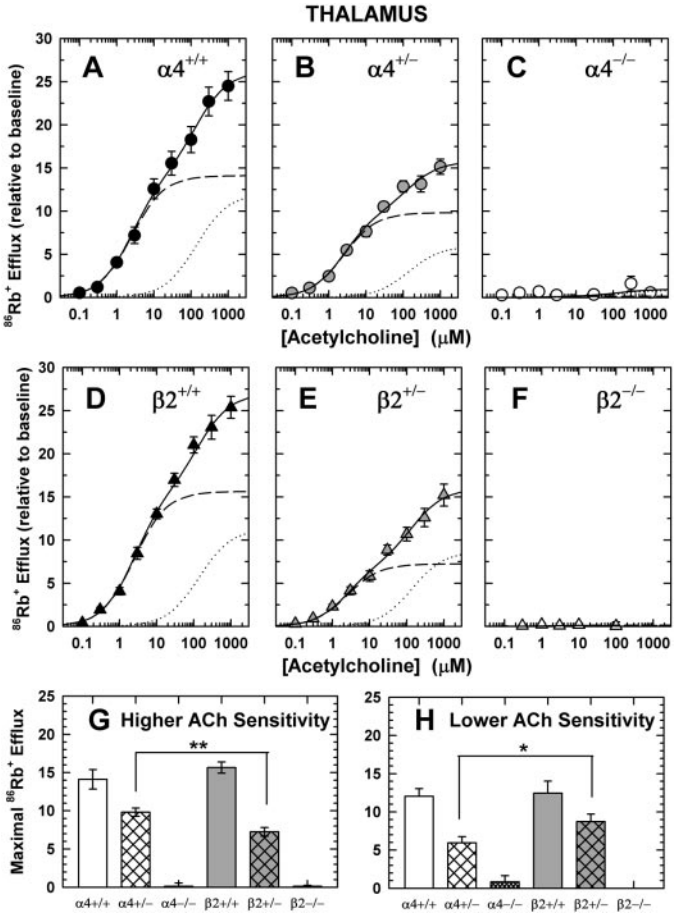

Fig. 3. ACh stimulation of $^{86}Rb^+$ efflux from thalamic synaptosomes. Crude synaptosomes were prepared from thalamus of $\alpha4^{+/+}$ (A; $n = 15$), $\alpha4^{+/-}$ (B; $n = 18$), $\alpha4^{-/-}$ (C; $n = 6$), $\beta2^{+/+}$ (D; $n = 13$), $\beta2^{+/-}$ (E; $n = 16$), and $\beta2^{-/-}$ (F; $n = 4$) mice. $^{86}Rb^+$ efflux was measured using the indicated [acetylcholine] as described under *Materials and Methods*. Data were fit to a two-component model, and the results of these curve fits are shown with the solid line. Higher and lower sensitivity components are indicated by the dashed and dotted lines, respectively. The effects of $\alpha4$ or $\beta2$ gene deletion on the maximal higher ACh sensitivity and lower ACh sensitivity components of $^{86}Rb^+$ efflux are shown in G and H, respectively A significant difference ($p < 0.05$) or ($p < 0.01$) between $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice are indicated with a single (*) or double (**) asterisk, respectively.

Thalamus. The effect of partial deletion of either the $\alpha4$ or $\beta2$ nAChR subunit on ACh-stimulated $^{86}Rb^+$ efflux was also examined in thalamic synaptosomes, and the data are presented in Fig. 3. The concentration-effect curves for $\alpha4$ and $\beta2$ wild-type mice were also biphasic. The higher sensitivity component comprised approximately 56% of total response in thalamic synaptosomes of wild-type mice (Fig. 3, A and D). No significant differences between $\alpha4^{+/+}$ and $\beta2^{+/+}$ were noted for either the higher sensitivity components ($\alpha4$, 14.14 ± 0.39; $\beta2$, 15.87 ± 0.42; $t_{26} = 1.01$, $p > 0.05$) or lower sensitivity components ($\alpha4$, 12.05 ± 0.99; $\beta2$, 12.24 ± 1.58; $t_{26} = 0.22$, $p > 0.05$).

Deletion of either subunit reduced both components by greater than 90%, and the residual activity was not significantly different from zero (Fig. 3, C and F). Similar to the results for cortex, total thalamic ACh-stimulated $^{86}Rb^+$ efflux was reduced approximately 40% after partial deletion of either the $\alpha4$ ($\alpha4^{+/-}$) or $\beta2$ ($\beta2^{+/-}$) subunit. Also similar to the results with cortex, when the ACh concentration effect curves were resolved into higher and lower sensitivity components differences between $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice were observed (Fig. 3, G and H). Partial deletion of the $\alpha4$ subunit ($\alpha4^{+/-}$) reduced the higher sensitivity $^{86}Rb^+$ efflux by 30 ± 3.5% and reduced lower sensitivity $^{86}Rb^+$ efflux by 51.0 ± 6.9%. In contrast, partial deletion of $\beta2$ subunit ($\beta2^{+/-}$) reduced the higher sensitivity $^{86}Rb^+$ efflux by 54 ± 2.4% and reduced lower sensitivity $^{86}Rb^+$ efflux by 21 ± 7.5%. Direct comparison of residual higher sensitivity components ($\alpha4^{+/-}$, 9.83 ± 0.41; $\beta2^{+/-}$, 7.23 ± 0.33; $t_{29} = 3.29$, $p < 0.05$) and lower sensitivity components ($\alpha4^{+/-}$, 5.96 ± 0.75; $\beta2^{+/-}$, 8.23 ± 0.60; $t_{29} = 2.17$, $p < 0.05$) revealed a differential effect of partial $\alpha4$ and $\beta2$ deletion in the respective heterozygotes. Thus, in thalamic synaptosomes partial deletion of the $\alpha4$ subunit ($\alpha4^{+/-}$) elicited a relatively larger decrease in the component of $^{86}Rb^+$ efflux with lower sensitivity to ACh, whereas partial deletion of the $\beta2$ subunit ($\beta2^{+/-}$) elicited a relatively larger decrease in the component with higher ACh sensitivity.

nAChR Expression in the Cortex and Thalamus of Mice Differing in $\alpha4$ and $\beta2$ Expression

The differential effects of partial $\alpha4$ or $\beta2$ nAChR subunit deletion on the higher and lower sensitivity components of ACh-stimulated $^{86}Rb^+$ efflux suggest that altering the relative expression of one of the subunits comprising the $\alpha4\beta2$*-nAChR subtype alters the functional properties of the receptor. To determine the effect of $\alpha4$ and $\beta2$ nAChR gene deletion on overall nAChR expression in the cortex and thalamus, ligand binding studies were performed using membranes prepared from wild-type ($\alpha4^{+/+}$or $\beta2^{+/+}$), heterozygote ($\alpha4^{+/-}$ or $\beta2^{+/-}$) and null mutant ($\alpha4^{-/-}$ or $\beta2^{-/-}$) mice.

[^{3}H]Epibatidine-Binding nAChRs. [^{3}H]Epibatidine binding was measured in membranes, extracts, and concentrated gradient fractions obtained from each $\alpha4$ and $\beta2$ genotype (Table 1). The samples were incubated with 2 μM αBgtx for 3 h before addition of [^{3}H]epibatidine to block binding at αBtx-sensitive sites. Binding took place using a saturating concentration of [^{3}H]epibatidine (2 nM).

Cortex. The density of cortical [^{3}H]epibatidine binding nAChRs (in femtomoles per milligram of protein) was 64.9 ± 7.2 in $\alpha4^{+/+}$ mice, 33.9 ± 3.3 in $\alpha4^{+/-}$ mice, and 2.5 ± 1.0 in $\alpha4^{-/-}$ mice (mean ± S.E.M. of four experiments) and 76.4 ±

3.9 in $\beta2^{+/+}$ mice, 41.4 ± 2.7 in $\beta2^{+/-}$ mice, and 2.0 ± 1.7 in $\beta2^{-/-}$ (mean ± S.E.M. of four experiments). The effect of gene deletion measured for cortical tissue using [^{3}H]epibatidine was comparable with that measured using ^{125}I-A85380 in independent experiments (Fig. 1). The specific activity of the 2% Triton X-100 was not different from that of the homogenate (Table 1). However, the specific activity of the concentrated gradient fraction samples from cortex was much higher than that of the 2% Triton X-100 extracts.

Thalamus. The density of thalamic [^{3}H]epibatidine binding nAChRs (in femtomoles per milligram of protein) was 187.8 ± 23.8 in $\alpha4^{+/+}$ mice, 105.0 ± 20.3 in $\alpha4^{+/-}$ mice, and 18.5 ± 9.2 in $\alpha4^{-/-}$ mice (mean ± S.E.M. of four experiments) and 177.4 ± 10.6 in $\beta2^{+/+}$ mice, 106.4 ± 15.2 in $\beta2^{+/-}$ mice, and 10.0 ± 3.4 in $\beta2^{-/-}$ mice. The effect of gene deletion measured using [^{3}H]epibatidine for thalamic tissue was comparable with that measured using ^{125}I-A85380 (Fig. 1). The specific activity of the extract was unchanged by 2% Triton X-100 extraction, and the values are reported in Table 1. As in the cortex, the specific activity of concentrated gradient fractions prepared from thalamus was much higher than that of the 2% Triton X-100 extracts.

Subunit Composition of Cortical and Thalamic [^{3}H]Epibatidine nAChRs. *Cortex.* The results described in the preceding paragraphs show that deletion of the $\alpha4$ or $\beta2$ subunit has measurable effects on overall nAChR expression, with an approximately 50% decrease in [^{3}H]epibatidine binding sites in both $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice. Samples were subsequently analyzed to determine whether gene deletion selectively affects the expression of some specific nAChR subtypes in heterozygotes and null mutants.

To quantify the relative contribution of each nicotinic subunit to [^{3}H]epibatidine binding in the cortex, quantitative immunoprecipitation experiments were performed using subunit-specific antibodies and [^{3}H]epibatidine labeled nAChRs on cortical tissue obtained from $\alpha4^{+/+}$, $\alpha4^{+/-}$, $\alpha4^{-/-}$, $\beta2^{+/+}$, $\beta2^{+/-}$, and $\beta2^{-/-}$ mice. Figure 4A shows the mean values of two separate experiments for each subunit in each genotype. The two major subtypes in both $\alpha4^{+/+}$ and $\beta2^{+/+}$ cortex were the $\alpha4\beta2$ (80–85% of all [^{3}H]epibatidine binding sites) and the $\alpha4\alpha5\beta2$ (10–15%). In heteterozygotes, the total number of $\alpha5$-containing subtypes (expressed as femtomoles per milligram of protein) was significantly reduced (from to 8.3 in $\alpha4^{+/+}$ to 4.8 in $\alpha4^{+/-}$ and from 8.4 in $\beta2^{+/+}$ to 4.8 in $\beta2^{+/-}$), but the percentage of [^{3}H]epibatidine-labeled $\alpha5$-containing receptors was the same in wild-type and heterozygotes (16–18%). The cortex also contained small amounts of $\alpha6$*-nAChRnd $\alpha3$*-nAChR, but the levels of these minor receptors were unchanged in the heterozygotes and they remained at the limit of our immunoprecipitation detection. The heterozygotes showed a selective decrease in the number of total [^{3}H]epibatidine binding sites, because of the decrease in the $\alpha4\beta2$-nAChR and $\alpha4\alpha5\beta2$-nAChR. However, the ratio of these two subtypes did not differ between heterozygotes and wild types.

Thalamus. The [^{3}H]epibatidine binding nAChRs expressed in thalamus of wild-type mice are slightly more heterogeneous than those expressed in the cortex. [^{3}H]Epibatidine binding sites containing solely $\alpha4$ and $\beta2$ subunits represent 70 to 80% of the total in $\alpha4$ and $\beta2$ wild types, and an additional 10% of the $\alpha4\beta2$*-nAChR also include the $\alpha5$ subunit. In addition, [^{3}H]epibatidine binding sites containing $\alpha3$ and $\beta4$ subunits comprise 6 to 7% in the sites in $\alpha4^{+/+}$ and 8 to 10% of the sites in the $\beta2^{+/+}$ thalamus (Fig. 4B). Approximately 5 to 6% of the [^{3}H]epibatidine binding nAChRs contained the $\alpha5$ subunit and 8 to 10% the $\beta3$ subunit in wild-type mice of both genotypes.

A decrease in $\alpha4$, $\alpha5$, and $\beta2$ subunit expression of approximately 50% from that in wild-types was observed in both $\alpha4^{+/-}$ and $\beta2^{+/-}$ thalamus, whereas the levels of the $\alpha3$ and $\beta4$ subunits were unchanged, and those of the $\beta3$ were only slightly decreased. To determine whether $\alpha3$, $\beta4$, and $\beta3$ subunits can be present in subtypes other than the $\alpha4\beta2$*-nAChR, parallel immunoprecipitation experiments were performed using $\alpha4^{-/-}$ and $\beta2^{-/-}$ mice. As shown in Fig. 4B, the only subtype present in the thalamus of the null mutant mice was $\alpha3\beta4\beta3$-nAChR, which was present at a level similar to that of wild type.

In conclusion, these immunoprecipitation data indicate that the majority of receptors in the thalamus (70–80%) are $\alpha4\beta2$-nAChR, 10% $\alpha4\alpha5\beta2$-nAChR, and approximately 10% $\alpha3\beta3\beta4$-nAChR.

Sucrose Gradient Analysis of nAChRs in the Cortex and Thalamus of $\alpha4$ and $\beta2$ Mice. To ascertain whether the $\alpha4$ and $\beta2$ subunits were incorporated into correctly assembled pentameric subtypes in mice, the nAChRs were fractionated using sucrose density-gradient centrifugation, and only the detergent-solubilized nAChRs of the correct size were analyzed by Western blots. Figure 5 shows the gradient profiles of cortical extracts prepared from $\alpha4^{+/+}$, $\alpha4^{+/-}$, $\beta2^{+/+}$, and $\beta2^{+/-}$ mice. After centrifugation, nAChRs within each fraction were captured using anti-$\alpha4$ and anti-$\beta2$ Ab-coated wells, and then they were quantitated by [^{3}H]epibatidine binding. The detergent solubilized nAChRs sedimented as a single species that was slightly larger than *T. californica* AChR monomers. These results clearly indicate that, although reduced in number, both the $\alpha4^{+/-}$ and $\beta2^{+/-}$ have a correct pentameric assembly. Moreover, these sedimentation experiments confirmed that the level

TABLE 1

Specific activities of the membrane, 2% Triton X-100 extracts, and concentrated gradient fractions

Values are the mean ± S.E.M. of four to six separate experiments for the cortex and three separate experiments for the thalamus.

	$\alpha4^{+/+}$	$\alpha4^{+/-}$	$\alpha4^{-/-}$	$\beta2^{+/+}$	$\beta2^{+/-}$	$\beta2^{-/-}$
	fmol/mg protein					
Cortex						
Membrane	64.9 ± 7.2	33.9 ± 3.3	2.5 ± 1.3	76.4 ± 3.9	41.4 ± 2.7	2.0 ± 1.7
2% Triton X-100	66.7 ± 10.6	34.6 ± 1.6	3.1 ± 0.8	76.5 ± 14.2	37.7 ± 4.6	1.1 ± 0.1
Concentrated gradient fraction	222 ± 32.8	116 ± 17.1	12.1 ± 3.9	247.5 ± 21.3	123.8 ± 12.3	2.4 ± 2.0
Thalamus						
Membrane	187.8 ± 23.8	105.0 ± 20.3	18.5 ± 9.2	177.4 ± 10.6,	106.4 ± 15.2	10.0 ± 3.4
2% Triton X-100	149.5 ± 26.5	89.9 ± 19.9	11.8 ± 4.0	170.3 ± 27.3	90.5 ± 2.3	9.5 ± 5.5
Concentrated gradient fraction	227.1 ± 30.5	138.8 ± 13.1	25.75 ± 2.3	288.6 ± 21,94	185.4 ± 17.2	22.3 ± 1.3

of expression of assembled $\alpha4\beta2$-nAChR was lower in heterozygotes, consistent with the immunoprecipitation results described above (Fig. 4A).

Qualitatively similar results were obtained on thalamic nAChRs separated on sucrose gradient (data not shown).

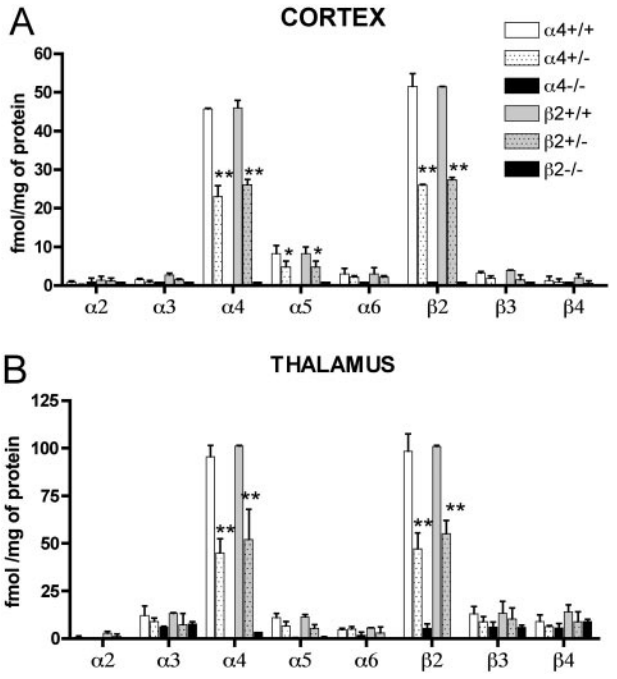

Fig. 4. Immunoprecipitation analysis of the subunit content of the nAChRs labeled with 2 nM [^{3}H]epibatidine, expressed in the cortex (A) and thalamus (B) of the $\alpha4^{+/+}$, $\alpha4^{+/-}$, $\alpha4^{-/-}$, $\beta2^{+/+}$, $\beta2^{+/-}$, and $\beta2^{-/-}$ genotypes. Immunoprecipitation was carried out as described under *Materials and Methods* using saturating concentrations (20–30 μg) of anti-subunit Abs. The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. The results obtained with each Ab are expressed as femtomoles of immunoprecipitated [^{3}H]epibatidine labeled nAChR per milligram of protein. The results are the mean values ± S.E.M. of three experiments performed in duplicate for the cortex (A) and two experiments performed in triplicate for each antibody for the thalamus (B). The statistical analyses were made using Student's paired t test. The significance of the differences from controls are *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

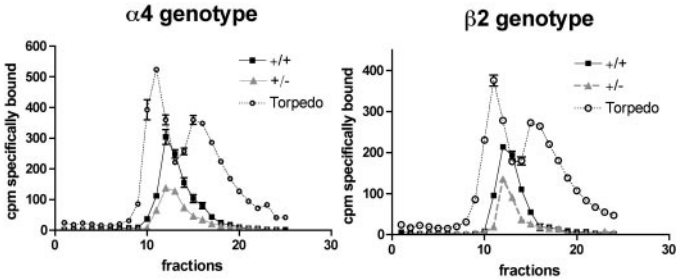

Fig. 5. Sucrose gradient analysis of cortical $\alpha4^{+/+}$, $\alpha4^{+/-}$, $\beta2^{+/+}$, and $\beta2^{+/-}$ genotypes. Then, 500 μl of 2% Triton X-100 extracts from each genotype were loaded onto a 5 to 20% (w/v) sucrose gradient in phosphate-buffered saline, pH 7.5, 0.1% Triton X-100, and 1 mM PMSF, and centrifuged for 14 h at 40,000 rpm in a Beckman rotor at 4°C as described under *Materials and Methods*. The fractions were collected, added to anti-$\alpha4$ or $\beta2$ Abs bound to microwells, left for 24 h, and then assayed for [^{3}H]epibatidine binding as described under *Materials and Methods*. As a standard, ^{125}I-αBgtx labeled *T. californica* AChRs were subjected to sucrose gradient centrifugation in parallel, the fractions were collected, and the radioactivity was determined by gamma counting. The peaks of monomer (9S) and dimer (11.5) *T. californica* AChR are shown.

Western Blot Analysis of $\alpha4$ and $\beta2$ Subunit Expression. As shown above and as reported previously, the major subtypes expressed in the cortex and thalamus are the $\alpha4\beta2$-nAChR and $\alpha4\alpha5\beta2$-nAChR subtypes. The immunoprecipitation studies clearly showed that the 50% reduction in [^{3}H]epibatidine binding sites in the $\alpha4^{+/-}$ and $\beta2^{+/-}$ genotypes is due to the loss of $\alpha4\beta2$-nAChR and $\alpha4\alpha5\beta2$-nAChR subtypes.

The specificity of our Abs were tested by incubating blots obtained from the cortex of wild-type and null mutant $\alpha4$ and $\beta2$ mice with Abs directed against the $\alpha4$ or $\beta2$ subunits after the electrophoresis of 10 μg of 2% Triton extract. As shown in Fig. 6, the anti-$\alpha4$ Ab recognized a peptide with a molecular mass of approximately 68 to 72 kDa (corresponding to the expected size of the $\alpha4$ subunit), in the extract taken from the wild-type but not $\alpha4^{-/-}$ mice (Fig. 6A, left), and the anti-$\beta2$ Ab recognized a band of 52 kDa (corresponding to the expected size of the $\beta2$ subunit) in the extract taken from the wild-type but not $\beta2^{-/-}$ mice (Fig. 6A, right).

The immunoprecipitation studies (Fig. 4) indicated that 8 to 10% of the thalamic [^{3}H]epibatidine binding nAChRs in both wild types contain the $\alpha3$ and $\beta4$ subunits. To exclude a possible antibody cross-reactivity, the $\alpha4\beta2$ subtype from $\beta2^{+/+}$ cortex was immunopurified over an affinity column with bound anti-$\beta2$ Abs and probed with the anti-$\alpha4$, -$\beta2$, -$\alpha5$, -$\alpha3$, -$\beta3$, and -$\beta4$ Abs. As shown in Fig. 6B, the purified $\alpha4\beta2$*-nAChR subtype was labeled by the anti-$\alpha4$, anti-$\alpha5$, and anti-$\beta2$ Abs but not by the anti-$\alpha3$, -$\beta3$, and -$\beta4$ Abs, further indicating that the Abs raised to $\alpha4$ and $\beta2$ subunits detect only the target subunit.

Cortex. Samples obtained from $\alpha4^{+/+}$, $\alpha4^{+/-}$, $\alpha4^{-/-}$ $\beta2^{+/+}$, $\beta2^{+/-}$, and $\beta2^{-/-}$ mice were analyzed by loading the same amount of protein of fully assembled receptors obtained after sucrose gradient centrifugation.

Figure 7A shows typical anti-$\alpha4$ and anti-$\beta2$ Ab labeling of

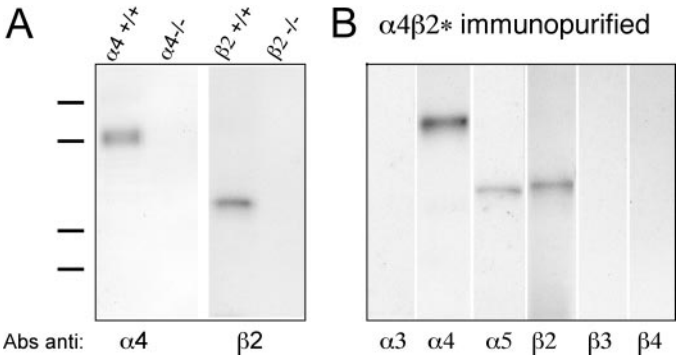

Fig. 6. Western blot analysis of antibody specificity in cortical extracts (A) and purified $\alpha4\beta2$* receptors from mouse cortex (B). A, samples obtained from the $\alpha4^{+/+}$, $\alpha4^{-/-}$, $\beta2^{+/+}$, and $\beta2^{-/-}$ genotypes were prepared as described under *Materials and Methods*. The proteins were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, probed with 1 to 2.5 μg/ml of the indicated primary Abs, and then incubated with the secondary Ab (anti-rabbit conjugated to peroxidase; dilution 1:40,000). The bound Abs were revealed by a chemiluminescent substrate (Pierce Chemical). The anti-$\alpha4$ Ab recognized a band of 68 to 70 kDa (corresponding to the expected size of the $\alpha4$ subunit) in the wild-type mice but not in the null mutant mice, and the anti-$\beta2$ Ab recognized a band of 52 kDa (corresponding to the expected size of the $\beta2$ subunit) in the wild-type mice but not in the null mutant mice. The molecular mass markers (top to bottom) are 97, 66, and 45, and 31 kDa. B, $\beta2$* receptors were purified as described under *Materials and Methods*. The proteins were concentrated, and then they were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, probed with 1 to 2.5 μg/ml of the indicated Abs, and processed as described above.

samples obtained by loading the same amount of protein after sucrose gradient centrifugation. Figure 7B shows the signal intensity for the $\alpha4$ and $\beta2$ subunits in the different genotypes expressed as the ratio of Ab labeling (taking the amount present in the samples from wild-type mice as 1) as shown by the results of four independent experiments using four separate sucrose gradients. The mean ± S.E.M. optical density ratios of the $\alpha4$ subunit in the $\alpha4$ genotype were 1.0 ± 0.0 in the $\alpha4^{+/+}$, 0.41 ± 0.02 in the $\alpha4^{+/-}$, and 0.04 ± 0.02 in the $\alpha4^{-/-}$, and those of the $\beta2$ subunit were1.0 ± 0.0 in the $\alpha4^{+/+}$, 0.50 ± 0.02 in the $\alpha4^{+/-}$, and 0.01 ± 0.006 in the $\alpha4^{-/-}$ genotypes.

A Mann-Whitney U-test indicated significant differences in the normalized optical densities of the $\alpha4^{+/+}$, $\alpha4^{+/-}$, and $\alpha4^{-/-}$ ($p < 0.001$) and the $\alpha4$ (0.41) and $\beta2$ (0.50) subunit ratios in $\alpha4^{+/-}$ were also significantly different ($*p = 0.038$).

Among the mice with different $\beta2$ expressions, the mean ± S.E.M. optical density ratios of the $\alpha4$ subunit were 1.0 ± 0.0 in the $\beta2^{+/+}$, 0.49 ± 0.01 in the $\beta2^{+/-}$, and 0.12 ± 0.02 in the $\beta2^{-/-}$ genotypes, and those of the $\beta2$ subunit were, respectively, 1.0 ± 0.0, 0.32 ± 0.006, and 0.02 ± 0.001. A Mann-Whitney U test showed significant difference in the normalized optical densities of the $\beta2^{+/+}$, $\beta2^{+/-}$, and $\beta2^{-/-}$ ($p < 0.001$) and that the $\alpha4$ (0.49) and $\beta2$ (0.32) subunit ratios in $\beta2^{+/-}$ were also significantly different ($***p = 0.0004$).

Immunoblotting experiments performed using constant protein for each of the six genotypes suggested that the ratio of the $\alpha4$ and $\beta2$ subunits was different in the preparations from the $\alpha4^{+/+}$, $\beta2^{+/+}$, $\alpha4^{+/-}$, and $\beta2^{+/-}$ mice, and so the $\alpha4$ and $\beta2$ subunits in the assembled receptors were measured using a different quantitative approach. The same number of binding sites of the sucrose gradient centrifugation fractions of the wild types (lanes 1 and 2) and the heterozygotes (lanes 3 and 4) were loaded onto the same gel, after which the blots were probed with anti-$\alpha4$ Ab and then stripped and probed with anti-$\beta2$ Ab (or vice versa). Figure 8A (left) shows the labeling of the same number of binding sites by the anti-$\alpha4$ (top) and the anti-$\beta2$ Ab (bottom) in the $\alpha4^{+/+}$ (lanes 1 and 2) and $\alpha4^{+/-}$ genotypes (lanes 3 and 4), and Fig. 8A (right) shows the labeling of the same number of binding sites by the anti-$\alpha4$ (top) and anti-$\beta2$ Ab (bottom) in the $\beta2^{+/+}$ (lanes 1 and 2) and $\beta2^{+/-}$ genotypes (lanes 3 and 4). Four separate sucrose gradient fraction samples for each genotype were measured using 1 or 2 fmol of binding sites per lane. The results, shown in Fig. 8B, are expressed as the ratio between the optical density of the heterozygotes divided by the optical density of the wild types. The heterozygote/wild-type ratios for $\alpha4^{+/-}/\alpha4^{+/+}$ were 0.85 ± 0.05 for the $\alpha4$ subunit Ab and 1.2 ± 0.05 for the $\beta2$ subunit Ab (Mann-Whitney U test; $**p = 0.0022$), whereas the ratios in the $\beta2^{+/-}/\beta2^{+/+}$ were 1.4 ± 0.1 and 0.78 ± 0.08 (Mann-Whitney U test; $**p = 0.0022$).

These data do not allow the assignment of the exact stoichiometry and ratios of the $\alpha4\beta2$-nAChR in wild-type and heterozygote cortices because the immunological signals of the $\alpha4$ and $\beta2$ subunits depend on the reactivity of the anti-$\alpha4$ and anti-$\beta2$ Abs, which may be different. However, the $\alpha4/\beta2$ ratios for the same number of binding sites were different for the $\alpha4^{+/-}$ and the $\beta2^{+/-}$ cortices, thus strongly suggesting that there is a difference in $\alpha4$ and $\beta2$ subunit content between $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice. That the heterozygote/wild-type ratios for the $\alpha4$ and $\beta2$ subunits were, respectively, 0.85 ± 0.05 and 1.2 ± 0.05

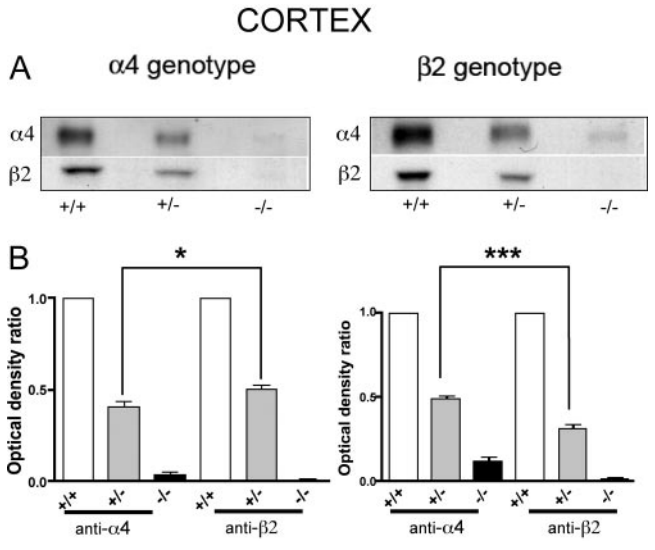

Fig. 7. Analysis of $\alpha4$ and $\beta2$ subunit content in cortical nAChRs prepared after gradient separation of the different genotypes. A, Western blot analysis of 10 μg of the concentrated fractions of the sucrose gradients from one representative experiment involving all genotype, tested for the presence of $\alpha4$ and $\beta2$ subunits. The proteins were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and processed as described in Fig. 6. B, optical density ratios of the $\alpha4$ and $\beta2$ subunits in the different genotypes. These ratios are the mean values ± S.E.M. of the Western blots of four separate experiments for each cortex genotype. The optical density ratio was calculated taking the optical density of the wild type as 1. The normalized optical density ratios of $\alpha4$ and $\beta2$ subunits in $\alpha4^{+/-}$ and $\beta2^{+/-}$ were significantly different (Mann-Whitney U test; *, $p = 0.038$; ***, $p = 0.0004$, respectively).

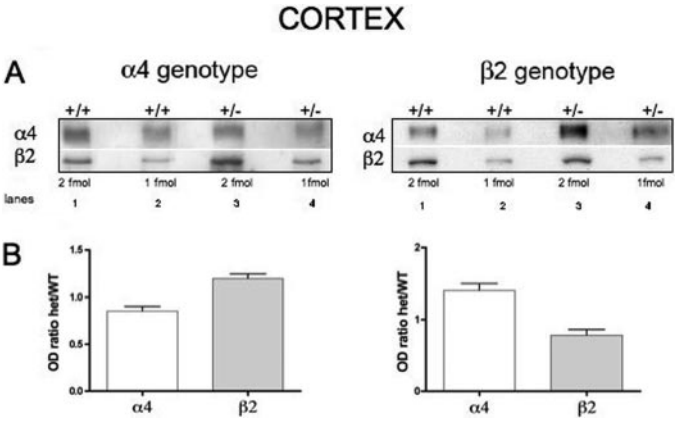

Fig. 8. Optical density ratios of the $\alpha4$ and $\beta2$ subunits in sucrose gradient fractions of cortex from the $\alpha4^{+/+}$ and $\alpha4^{+/-}$ and the $\beta2^{+/+}$ and $\beta2^{+/-}$ genotypes. A, 2 or 1 fmol in total of $\alpha4^{+/+}$ (lanes 1 and 2) or $\alpha4^{+/-}$ (lanes 3 and 4) of [^{3}H]epibatidine binding sites was loaded per lane. The samples were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and processed as described in Fig. 6. The same blots were first incubated with anti-$\alpha4$ Abs, and then they were stripped and incubated with anti-$\beta2$ Abs or vice versa. The developed films were acquired as described under *Materials and Methods*, and the images were analyzed using National Institutes of Health ImageJ software (National Technical Information Service). The pixel values of all of the images were transformed to optical density values by the program using the calibrated curve obtained by acquiring the calibrated tablet using the same parameters as those used for the images. The ratios between the optical densities of lane 3 and lane 1 and lane 4 and lane 2 were obtained for both $\alpha4$ and $\beta2$ Abs. B, mean values ± S.E.M. of optical density ratios of the $\alpha4$ and $\beta2$ subunits in the $\alpha4$ (left) and $\beta2$ genotypes (right) of heterozygote/wild-type mice in the $\alpha4$ genotype (left) and $\beta2$ genotypes (right) obtained from four separate genotype experiments. The cortex $\alpha4$ and $\beta2$ subunit ratios in the $\alpha4^{+/-}/\alpha4^{+/+}$ and $\beta2^{+/-}/\beta2^{+/+}$ genotypes were statistically significant (Mann-Whitney U test; **, $p = 0.0022$ for both).

in the $\alpha4$ genotype indicates that there are more receptors with the possible $(\alpha4)_2(\beta2)_3$ stoichiometry in $\alpha4^{+/-}$ than in $\alpha4^{+/+}$, and that the ratios for the $\alpha4$ and $\beta2$ subunits were, respectively, 1.4 ± 0.1 and 0.78 ± 0.08 indicates that a higher content of receptors with the $(\alpha4)_3(\beta2)_2$ subunit stoichiometry in the $\beta2^{+/-}$ than in the $\beta2^{+/+}$.

Thalamus. Sucrose gradient fractions prepared from thalamic tissue were also analyzed by quantitative Western blotting. The results of three independent experiments using two separate sucrose gradients are shown in Fig. 9.

In the mice with different $\alpha4$ expression, the mean ± S.E.M. optical density ratios for the $\alpha4$ subunit were 1.0 ± 0.0 in $\alpha4^{+/+}$, 0.46 ± 0.03 in $\alpha4^{+/-}$, and 0.04 ± 0.01 in $\alpha4^{-/-}$, and those of the $\beta2$ subunit were 1.0 ± 0.0 in $\alpha4^{+/+}$, 0.54 ± 0.04 in $\alpha4^{+/-}$, and 0.03 ± 0.002 in the $\alpha4^{-/-}$. A Mann-Whitney U test showed significant difference in the normalized optical densities of $\alpha4^{+/+}$, $\alpha4^{+/-}$, and $\alpha4^{-/-}$ mice ($p < 0.01$), and the $\alpha4$ (0.46) and $\beta2$ (0.54) subunit ratios ($\alpha4^{+/-}/\alpha4^{+/+}$) were not significantly different from each other.

In the mice with different $\beta2$ expression, the mean ± S.E.M. optical density ratios for the $\alpha4$ subunit were 1.0 ± 0.0 in $\beta2^{+/+}$, 0.56 ± 0.07 in $\beta2^{+/-}$, and 0.12 ± 0.04 in $\beta2^{-/-}$, and those of the $\beta2$ subunit were 1.0 ± 0.0, 0.33 ± 0.002, and 0.02 ± 0.002, respectively. A Mann-Whitney U test showed significant differences in the normalized optical densities of both the $\alpha4$ and $\beta2$ subunits in the $\beta2^{+/+}$, $\beta2^{+/-}$, and $\beta2^{-/-}$ mice ($p < 0.001$). The relative expression of the $\alpha4$ (0.56) and $\beta2$ (0.33) subunits in heterozygotes relative to wild type were differentially affected by partial deletion of the $\beta2$ gene ($\beta2^{+/-}$), and it differed significantly from each other ($*p = 0.015$).

Additional blot experiments were performed on thalamus in which the same number of binding sites from sucrose gradient fractions was loaded onto the same gel for direct comparison of the ratio of $\alpha4$ and $\beta2$ subunits in the wild types and heterozygotes. The blots were probed with anti-$\alpha4$ Ab, and then they were stripped and probed with anti-$\beta2$ Abs (or vice versa). Figure 10A (left) shows the labeling of the same number of binding sites by anti-$\alpha4$ (top) and anti-$\beta2$ Ab (bottom) in the $\alpha4^{+/+}$ (lanes 1 and 2) and $\alpha4^{+/-}$ genotypes (lanes 3 and 4), and Fig. 10A (right) shows the labeling of the same number of binding sites by the anti-$\alpha4$ (top) and anti-$\beta2$ Ab (bottom) in $\beta2^{+/+}$ (lanes 1 and 2) and $\beta2^{+/-}$ genotypes (lanes 3 and 4). Three separate sucrose gradient fraction samples were tested for each genotype.

The results, shown in Fig. 10B, are expressed as the ratio between the optical density of the heterozygote and wild-type genotype measured using 1 or 2 fmol of binding sites per lane. The mean optical density ratios ± S.E.M. for the $\alpha4^{+/-}/\alpha4^{+/+}$ ratios were 0.80 ± 0.05 for the $\alpha4$ subunit and 1.53 ± 0.09 for the $\beta2$ subunit (Mann-Whitney U test; $*p = 0.029$), and those for the $\beta2^{+/-}/\beta2^{+/+}$ genotype were 1.44 ± 0.09 for the $\alpha4$ subunit and 0.9 ± 0.1 for the $\beta2$ subunit (Mann-Whitney U test; $**p = 0.0079$).

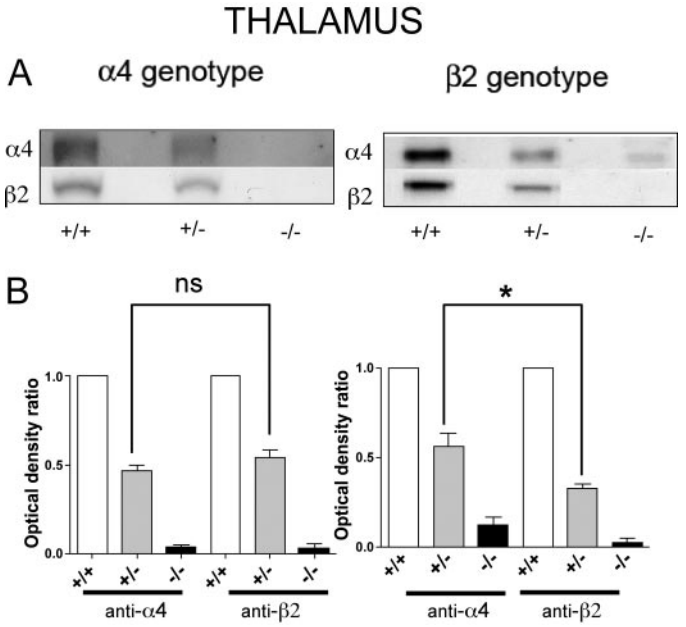

Fig. 9. Analysis of $\alpha4$ and $\beta2$ subunit content in thalamic nAChRs prepared after gradient separation of the different genotypes. A, Western blot analysis of 7 μg of the concentrated sucrose density gradient fractions from one representative experiment involving all genotypes and tested for the presence of $\alpha4$ and $\beta2$ subunits. The proteins were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and processed as described in Fig. 6. B, optical density ratios of the $\alpha4$ and $\beta2$ subunits in the different genotypes. These ratios are the mean ± S.E.M. of Western blots of three separate experiments for each genotype. The optical density ratio was calculated by taking the optical density of the wild-type as 1. The normalized optical density ratios of the $\alpha4$ and $\beta2$ subunits were not statistically significant (ns) in the $\alpha4^{+/-}$ mice, but $\beta2^{+/-}$ was significantly different in the $\beta2^{+/-}$ mice (Mann-Whitney U test; $*$, $p = 0.015$).

Discussion

As reported previously (Marks et al., 1999; Brown et al., 2007) and confirmed here (Figs. 2 and 3), ACh-stimulated $^{86}Rb^+$ efflux from cortical and thalamic synaptosomes is biphasic, with components displaying high sensitivity ($EC_{50} \approx 2$ μM) and low sensitivity ($EC_{50} \approx 100$ μM) to activation by ACh. Both components in these regions are primarily $\alpha4\beta2^*$-nAChR, because deletion of either $\alpha4$ (Marks et al., 2007) or $\beta2$ (Marks et al., 1999, 2000) subunit virtually eliminated

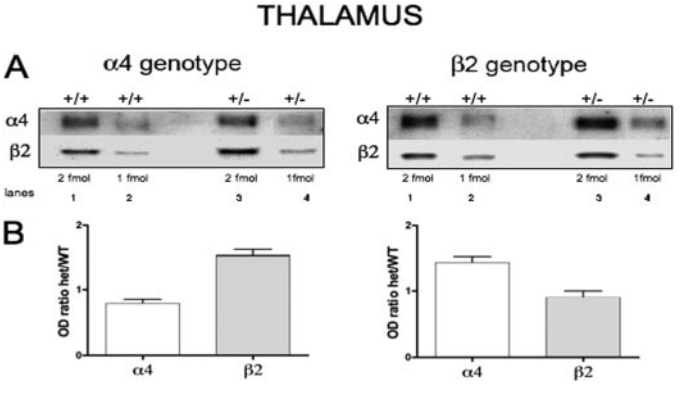

Fig. 10. Optical density ratios of the $\alpha4$ and $\beta2$ subunits in sucrose gradient fractions of thalamus from the $\alpha4^{+/+}$ and $\alpha4^{+/-}$, and the $\beta2^{+/+}$ and $\beta2^{+/-}$ genotypes. A, 2 or 1 fmol in total of [^{3}H]epibatidine binding sites for $\alpha4^{+/+}$ (lanes 1 and 2) or $\alpha4^{+/-}$ (lanes 3 and 4) binding sites was loaded per lane. The samples were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and processed as described in Fig. 8. The same blots were first incubated with anti-$\alpha4$ Abs, and then they were stripped and incubated with anti-$\beta2$ Abs or vice versa. The samples were processed, analyzed, and expressed as described in Fig. 8A. B, mean values ± S.E.M. of optical density ratios of the $\alpha4$ and $\beta2$ subunits in the $\alpha4$ (left) and $\beta2$ genotypes (right) of heterozygote/wild type obtained from three separate genotype experiments. The thalamic heterozygote/wild-type $\alpha4$ and $\beta2$ subunit ratios were statistically significant in the $\alpha4^{+/-}/\alpha4^{+/+}$ and $\beta2^{+/-}/\beta2^{+/+}$ genotypes (Mann-Whitney U test; $*$, $P = 0.029$ and $**$, $p = 0.0079$, respectively).

them. $\alpha4\beta2$-nAChRs expressed in *Xenopus laevis* oocytes or embryonic kidney cells also display biphasic ACh concentration-effect curves with EC_{50} values comparable with those measured for $^{86}Rb^+$ efflux (Zwart and Vijverberg, 1998; Nelson et al., 2003; Khiroug et al., 2004; Briggs et al., 2006; Moroni et al., 2006). The relative expression of the two components can be modified by injecting differing ratios of the mRNAs for the $\alpha4$ and $\beta2$ subunits. Oocytes injected with a large excess of $\beta2$ mRNA express primarily the high sensitivity responses, whereas oocytes injected with a large excess of $\alpha4$ mRNA express primarily the low sensitivity responses (Zwart and Vijverberg, 1998; Nelson et al., 2003; Moroni et al., 2006), leading to the postulate that differential sensitivity to agonist stimulation results from differences in $\alpha4\beta2$-nAChR subunit stoichiometry [$(\alpha4)_2(\beta2)_3$ for high and $(\alpha4)_3(\beta2)_2$ for low]. Compelling evidence that supports the assertion that subunit stoichiometry influences sensitivity to agonists was obtained in studies with linked $\alpha4/\beta2$ subunits that were expressed with either an additional $\beta2$ or $\alpha4$ subunit (Zhou et al., 2003; Tapia et al., 2007). These manipulations resulted in expression of either high- or low-sensitivity receptors, respectively

Because alteration of relative $\alpha4$ and $\beta2$ expression changes receptor stoichiometry in heterologous expression systems, heterozygous $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice were used in current study to evaluate the effects of manipulation of mouse brain mRNA levels in vivo on protein expression and receptor function. Previously, we reported that deletion or partial deletion of either $\alpha4$ or $\beta2$ reduced mRNA levels for the target subunit without affecting mRNA levels of the complementary subunit (Whiteaker et al., 2006). Here, we report that partial deletion of either the $\alpha4$ ($\alpha4^{+/-}$)or $\beta2$ ($\beta2^{+/-}$) subunit gene decreased the expression of $\alpha4\beta2$*-nAChRs measured by ^{125}I-A85380 binding to synaptosomal membranes (Fig. 1) and [^{3}H]epibatidine binding precipitated by both the anti-$\alpha4$ and the anti-$\beta2$ antibodies (Table 1; Fig. 4) in both the cortex and thalamus by 50%. Furthermore, this decrease in binding reflects a decrease in the number of properly assembled receptors determined by sucrose gradient centrifugation (Fig. 5).

In the current study, we observed that both components of ACh-stimulated $^{86}Rb^+$ efflux were reduced in $\alpha4^{+/-}$ and $\beta2^{+/-}$ cortex and thalamus. If the stoichiometry of the $\alpha4\beta2$*-nAChR was unaffected by partial deletion of either the $\alpha4$ or $\beta2$ subunit, the proportion of these two components should remain unchanged in tissue obtained from the heterozygous mice. However, partial deletion of the $\alpha4$ subunit [this should favor the formation of $(\alpha4)_2(\beta2)_3$ nAChRs] resulted in a relatively larger decrease in the lower sensitivity component, whereas partial deletion of the $\beta2$ subunit [this should favor the formation of $(\alpha4)_3(\beta2)_2$ nAChRs] resulted in a relatively larger decrease in the higher sensitivity component. These results are consistent with the hypothesis that the stoichiometry of $\alpha4\beta2$*-nAChR is affected by the relative expression of $\alpha4$ and $\beta2$ mRNA in vivo, and they are in agreement with the results observed in heterologous expression systems.

To test whether subunit expression was actually changed by partial gene deletion, Western blots with subunit specific Abs were used to analyze the subunit content of correctly assembled pentameric receptors fractionated on sucrose gradients. No signal was obtained using extracts from $\alpha4^{-/-}$ or $\beta2^{-/-}$ mice, thereby demonstrating that the Abs used in this study were specific. Such analysis is essential because specificity of different batches of polyclonal Abs prepared in different rabbits can vary between techniques and even when using the same technique (Gotti et al., 2006). The extent of the deletion on [^{3}H]epibatidine binding was greater for the $\beta2$ subunit, which was absent from the cortex and thalamus of $\alpha4^{-/-}$ mice, whereas a residual 10% of $\alpha4$ immunoreactivity was still present in $\beta2^{-/-}$ mice. These results are generally consistent with our histochemical analyses of these same gene deletions using different antibodies (Whiteaker et al., 2006).

Except for the target $\alpha4$ and $\beta2$ genes and also the $\alpha5$ gene, partial or complete deletion of either the $\alpha4$ or $\beta2$ subunit did not alter the expression profile for other cortical or thalamic nAChR proteins (Fig. 4). If stoichiometry were unchanged by partial gene deletion, the ratios of $\alpha4$:$\beta2$ in the $\alpha4^{+/-}$ and $\beta2^{+/-}$ would be expected to be the same as those seen in the wild-type control mice. One definitive way of determining receptor stoichiometry is to measure the number of $\alpha4$ and $\beta2$ subunits in fully assembled receptors by metabolically labeling the proteins as has been done in chick and rat $\alpha4\beta2$ subtypes expressed in heterologous systems (Anand et al., 1991; Nelson et al., 2003). However, this is not feasible in vivo. As an alternative approach, assembled receptors were quantitated immunologically. Labeling native receptors with anti-$\alpha4$ and anti-$\beta2$ Abs cannot define the stoichiometry of wild-type receptors because the immunolabeling depends on the avidity of the Abs and because the relationship between the amount of subunit and signal intensity is linear only in a narrow window. Considering these limitations, careful quantitation of Ab labeling intensity using the same amount of protein or the same number of binding sites from wild-type and heterozygous mice yielded data that suggest that the ratio of $\alpha4$ and $\beta2$ subunit protein in assembled $\alpha4\beta2$-nAChRs differs between control and heterozygous mutant mice. In both cortex and thalamus, relative expression of the $\alpha4$ subunit is lower than that of the $\beta2$ subunit in $\alpha4^{+/-}$ genotype, whereas relative expression of the $\alpha4$ subunit is higher than that of the $\beta2$ in $\beta2^{+/-}$ mice. These results are consistent with an altered stoichiometry of $\alpha4\beta2$*-nAChR after partial gene deletion such that $\alpha4^{+/-}$ have more receptors with a stoichiometry of $(\alpha4)_2(\beta2)_3$ and $\beta2^{+/-}$ have more receptors with a stoichiometry of $(\alpha4)_3(\beta2)_2$.

The consequences of partial $\alpha4$ or $\beta2$ deletion determined with the immunochemical studies parallel those of partial gene deletion on $^{86}Rb^+$ efflux. The relatively larger decrease in the lower sensitivity component of ACh-stimulated $^{86}Rb^+$ efflux in $\alpha4^{+/-}$ and a relatively larger decrease in the higher sensitivity component in the $\beta2^{+/-}$ is consistent with the changes in relative subunit expression in the heterozygous mice determined by Ab labeling.

An important consideration associated with studying brain is that many different nAChRs might be present. We chose cortex and thalamus for the studies reported here because gene deletion experiments showed that almost all [^{3}H]epibatidine binding sites (Marks et al., 2006, 2007) and $^{86}Rb^+$ efflux (Marks et al., 2000, 2007) in these brain regions are $\alpha4\beta2$, with a small fraction of $\alpha4\alpha5\beta2$ (Brown et al., 2007). The immunological studies (Fig. 4) support this postulate. Most of the signal in cortex is clearly $\alpha4\beta2$; $\alpha5$ subunits that coassemble with $\alpha4$ and $\beta2$ represent 10 to 15% of the total $\alpha4\beta2$*-nAChR binding sites in thalamus (Fig. 4; Gotti et al., 2006). These $\alpha4\beta2\alpha5$-nAChRs seem to mediate more than 10

to 15% of total ACh-stimulated ion flux in thalamus, but very little of this response in cortex (Brown et al., 2007).

The evidence presented here strongly suggests that $\alpha4\beta2$-nAChRs differing in subunit stoichiometry exist in vivo. The higher and lower sensitivity components show different pharmacological profiles for both agonists and antagonists in brain (Briggs et al., 2006; Marks et al., 1999) and in heterologous expression systems (Moroni et al., 2006). The two components also differ in calcium permeability (Tapia et al., 2007) and in up-regulation after long-term nicotine exposure both in vitro (Buisson and Bertrand, 2001; Nelson et al., 2003) and treatment in vivo (Marks et al., 2004). The relative expression of the two components also differs across mouse brain regions (Marks et al., 2000, 2007), suggesting that relative effects of nicotinic drugs could vary among brain regions. Furthermore, using heterologous expression systems differences in the either untranslated regions of the gene (Briggs et al., 2006) or naturally occurring polymorphisms in the coding regions (Kim et al., 2003) affect relative expression $\alpha4\beta2$-nAChR with higher or lower ACh sensitivity. The demonstration that natively expressed $\alpha4\beta2$-nAChR very likely have alternative stoichiometries, which can be influenced by the relative level of expression of mRNA encoding the $\alpha4$ and $\beta2$ subunits, supports the results obtained using heterologous expression systems and implies that these alternatively assembled receptors could have important consequences in vivo.

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