

Original Article

Pharmacological and immunochemical characterization of $\alpha 2^*$ nicotinic acetylcholine receptors (nAChRs) in mouse brain

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Aim: $\alpha 2$ nAChR subunit mRNA expression in mice is most intense in the olfactory bulbs and interpeduncular nucleus. We aimed to investigate the properties of $\alpha 2^*$ nAChRs in these mouse brain regions.

Methods: $\alpha 2$ nAChR subunit-null mutant mice were engineered. Pharmacological and immunoprecipitation studies were used to determine the composition of $\alpha 2$ subunit-containing ($\alpha 2^*$) nAChRs in these two regions.

Results: [¹²⁵I]Epibatidine (200 pmol/L) autoradiography and saturation binding demonstrated that $\alpha 2$ deletion reduces nAChR expression in both olfactory bulbs and interpeduncular nucleus (by 4.8 ± 1.7 and 92 ± 26 fmol·mg⁻¹ protein, respectively). Pharmacological characterization using the $\beta 2$ -selective drug A85380 to inhibit [¹²⁵I]epibatidine binding proved inconclusive, so immunoprecipitation methods were used to further characterize $\alpha 2^*$ nAChRs. Protocols were established to immunoprecipitate $\beta 2$ and $\beta 4$ nAChRs. Immunoprecipitation specificity was ascertained using tissue from $\beta 2$ - and $\beta 4$ -null mutant mice, and efficacy was good (>90% of $\beta 2^*$ and >80% of $\beta 4^*$ nAChRs were routinely recovered).

Conclusion: Immunoprecipitation experiments indicated that interpeduncular nucleus $\alpha 2^*$ nAChRs predominantly contain $\beta 2$ subunits, while those in olfactory bulbs contain mainly $\beta 4$ subunits. In addition, the immunoprecipitation evidence indicated that both nuclei, but especially the interpeduncular nucleus, express nAChR complexes containing both $\beta 2$ and $\beta 4$ subunits.

Keywords: nicotinic acetylcholine receptors; receptor subtypes; radioligand assay; immunoprecipitation

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Introduction

nAChRs mediate the physiological effects of exogenous nicotine. They also play critical physiological roles throughout the brain and body by mediating cholinergic excitatory neurotransmission, modulating the release of neurotransmitters, and having longer-term effects on, for example, gene expression and cellular connections^[1]. Mammalian nAChR subunits are derived from a family of sixteen different genes ($\alpha 1$ – $\alpha 7$, $\alpha 9$ – $\alpha 10$, $\beta 1$ – $\beta 4$, γ , δ , and ϵ) which have distinctive distributions, and assemble into pentameric receptors. Importantly, the combinations and orders of nAChR subunits within functional pentamers dictate nAChR subtype properties^[2]. The properties include pharmacology and

functional features, such as channel kinetics. This is in part because, although homologous, subunit proteins and the interfaces between them are structurally (sometimes subtly) unique.

The $\alpha 2$ subunit was among the first non-muscle nAChR subunits to be cloned and heterologously expressed in a functional subtype, with the $\beta 2$ subunit^[3–5]. However, studies into $\alpha 2$ subunit containing ($\alpha 2^*$) nAChRs were soon overtaken in number by those into other subtypes. Possible reasons include the highly-restricted expression of $\alpha 2^*$ nAChRs in commonly used rodent models^[6–8], the pharmacological similarity of $\alpha 2^*$ nAChRs to much more prevalent $\alpha 4^*$ nAChRs^[9], which makes $\alpha 2^*$ nAChRs hard to identify pharmacologically (although DH β E may provide a useful pharmacological probe^[10, 11]), and even the lack of specific pharmacological probes and/or, until extremely recently^[12], antibodies for $\alpha 2^*$ nAChRs.

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However, more-recent evidence suggests that $\alpha 2^*$ nAChRs may deserve more attention. An mRNA *in situ* hybridization study in the primate *Macaca mulatta* demonstrated that $\alpha 2$ mRNA expression is much more prevalent in this primate model, with the implication that this may be the case in other primates, including humans^[13]. This finding was recently reinforced by an immunochemical study demonstrating that approximately 10% of human temporal cortex nAChRs contain $\alpha 2$ subunits^[14]. $\alpha 2^*$ nAChRs do seem to have important physiological roles. In humans, a mutant (I279N) $\alpha 2$ subunit has been identified, which forms nAChRs with increased agonist sensitivity and causes a form of familial epilepsy^[15], and a single nucleotide polymorphism study has provided preliminary evidence of a link to overweight/obesity in humans^[16]. Further, an $\alpha 2$ subunit-null mouse model has been used to demonstrate a role for $\alpha 2^*$ nAChRs in nicotine-induced modulation of long-term potentiation in the mouse hippocampal CA1 region, which may underlie some of the cognitive effects of nicotine^[17].

Earlier work demonstrated that $\alpha 2\beta 2$ and $\alpha 2\beta 4$ nAChRs have widely-divergent pharmacological properties^[9]. Despite the renewed interest in $\alpha 2^*$ nAChR investigations, little is known about the relative contributions of $\beta 2$ and $\beta 4$ subunit partners in native $\alpha 2^*$ nAChR subtypes. This study employs the $\alpha 2$ subunit-null mouse model, in combination with pharmacological and immunoprecipitation studies, to address this question.

Materials and methods

Animals Wild-type C57BL/6 mice, and lines engineered to contain a null mutation in the $\alpha 2$, $\beta 2$, and $\beta 4$ nAChR subunit genes were bred at the Institute for Behavioral Genetics, and housed five per cage. All mutant mouse lines were backcrossed onto the C57BL/6 background (minimum of six generations). The vivarium was maintained on a 12 h light/dark cycle (lights on 0700 to 1900 h), and mice were given free access to food and water. Male mice were used throughout this study. All procedures used in this study were approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder (CO, USA).

Materials [¹²⁵I]Epibatidine (specific activity, 2200 Ci/mmol) was obtained from PerkinElmer Lifesciences (Boston, MA). (–)-Nicotine bitartrate was bought from BDH Chemicals (Poole, UK). A85380 was supplied by Research Biochemicals (Natick, MA). All other supplies were purchased from Sigma (St Louis, MO), unless specifically noted.

Membrane preparation Each mouse was euthanized by

cervical dislocation. The brain was removed from the skull and placed on an ice-cold platform. Regions of interest were dissected then homogenized in ice-cold hypotonic buffer (mmol/L: NaCl, 14.4; KCl, 0.2; CaCl₂, 0.2; MgSO₄, 0.1; HEPES 2; pH=7.5) using a glass-PTFE tissue grinder^[18]. Particulate fractions were obtained by centrifugation at 25 000×g (15 min, 4 °C). The pellets were resuspended in fresh homogenization buffer, incubated at 22 °C for 10 min, then harvested by centrifugation as before. Each pellet was washed twice more by resuspension/centrifugation, then stored (in pellet form under homogenization buffer) at –80 °C until used.

[¹²⁵I]Epibatidine saturation binding to membranes

Binding of [¹²⁵I]-epibatidine was quantified as previously described^[19]. Incubations were performed in 96-well polystyrene plates, in 30 μ L of binding buffer (mmol/L: NaCl, 144; KCl, 1.5; CaCl₂, 2; MgSO₄, 1; HEPES, 20; pH=7.5). Plates were covered to minimize evaporation during incubation, and all incubations progressed for 2 h at 22 °C. Saturation binding experiments were performed for membrane preparations from each brain region, using ligand concentrations ranging between approximately 5–200 pmol/L. Binding reactions were terminated by filtration of samples onto a single thickness of polyethyleneimine-soaked (0.5% w/v in binding buffer) GF/F glass fiber filters (Gelman Sciences, Ann Arbor, MI, USA) using an Inotech Cell Harvester (Inotech, Rockville, MD, USA). Samples were subsequently washed six times with ice-cold binding buffer. Total and non-specific [in the presence of 1 mmol/L (–)-nicotine tartrate] binding were determined in triplicate for each [¹²⁵I]epibatidine concentration. Bound ligand was quantified by gamma counting at 85% efficiency. At the lower concentrations, a significant proportion (up to 20%) of ligand bound to the tissue. Free [¹²⁵I]epibatidine concentrations were estimated by correcting for the amount of ligand bound to tissue, and these corrected concentrations were used to calculate K_D values for [¹²⁵I]epibatidine binding in each brain region.

Inhibition of [¹²⁵I]-epibatidine binding to membranes

Inhibition binding experiments were performed using 200 pmol/L [¹²⁵I]epibatidine, in the same assay format as described for saturation binding. The amount of membrane protein added was chosen to produce maximum binding of ligand to the tissue of approximately 40 Bq/well (less than 10% of total ligand added, minimizing the effects of ligand depletion). Various concentrations of competing drugs were included in duplicate wells. Non-specific binding was determined in the presence of 1 mmol/L (–)-nicotine tartrate for each experiment.

Immunoprecipitation of nAChR subunits Immunoprecipitation of [125 I]epibatidine sites with nAChR subunit specific monoclonal antibodies (mAbs) was performed as previously described^[20]. Olfactory bulbs and IPNs from single mice were homogenized using a glass-PTFE hand homogenizer, in assay buffer (50 mmol/L NaCl, 50 mmol/L sodium phosphate, 2 mmol/L EDTA, 2 mmol/L EGTA, and 2 mmol/L phenylmethylsulfonylfluoride; pH 7.4), and centrifuged for 12 min at 20 000×g. Pellets were resuspended and solubilized in assay buffer supplemented with 10 µg/mL each of leupeptin, pepstatin A, and aprotinin with 2% Triton X-100 for 60 min at room temperature (1 mL per sample). This was followed by centrifugation for 15 min at 25 000×g and collection of the supernatant. Protein G CPG magnetic beads (2 µL of bead volume; PureBiotech LLC, Middlesex, NJ) were added to the samples, along with either mAb 295 or mAb 337 (against $\beta 2$ and $\beta 4$ nAChR subunits, respectively). The antibody-bead-Triton extract mixtures were rotated for 8 h at 4 °C. Initially, to test antibody potencies, ranges of antibody concentrations were tested (mAb 295, 0.003–3 µg/mL; mAb 337, 0.005–50 µg/mL). Maximally-effective concentrations (10 µg/mL of each antibody) were used for all subsequent experiments. The assays were terminated by magnetic sedimentation. At this stage, supernatants were removed and placed into separate tubes, and the pellets were washed (three resuspensions and resedimentations in fresh assay buffer, 4 °C), with a final resuspension into 1 mL of buffer. Both the supernatant and pellet were then assayed for [125 I]epibatidine binding sites as described for membrane preparations, previously. Before filtration, [125 I]epibatidine binding sites in the samples were first precipitated by addition of polyethylene glycol (average molecular weight, 8000; PEG-8000) to a final concentration of 20% and then collected by filtration onto polyethylenimine-soaked [0.5% (w/v)] Gelman GF/F filter paper (Gelman Instrument Co., Ann Arbor, MI) using a 48-well format Inotech filtration apparatus (Inotech Biosystems, Rockville, MD). For all samples the radioactivity was measured by gamma counting at 85% efficiency. Nonspecific absorption was measured in the absence of the appropriate mAb. Control (no mAb) conditions were tested in parallel for each sample, and in all cases the sum of supernatant and pellet binding matched the supernatant binding in the control samples. Nonspecific binding was defined using 1 mmol/L nicotine. No [125 I]epibatidine binding sites were detected in any of the no-mAb control pellets. In addition, the specificity of mAb 295 and mAb 337 for their target subunits ($\beta 2$ and $\beta 4$, respectively) was tested using the corresponding subunit-null mutant mice. Brains from eight wild-type mice and four each of $\beta 2^{-/-}$ and

$\beta 4^{-/-}$ genotypes were dissected into 14 regions on an ice-cold platform. mAb 295 and 337 immunoabsorption assays were then done as described above, using tissue from mice lacking the target subunit for each antibody.

Protein determination Protein was determined using the method of Lowry^[21], with bovine serum albumin as standard.

Data analysis Radioactive counts were calculated by subtracting blank samples containing no mAb and normalizing to protein concentration. All values are expressed as the mean±SEM of the indicated number of animals. Statistical comparisons of data shown in the figures were performed using ANOVA with *post hoc* testing.

Results

[125 I]Epibatidine saturation filtration binding, effects of $\alpha 2$ -null mutation Previous publications indicated that, in mice, $\alpha 2$ nAChR subunit mRNA is most-concentrated in the olfactory bulbs and dorsal interpeduncular nucleus^[7, 8]. Accordingly, we compared nAChR expression in these two brain regions between $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice, using the general nAChR radioligand [125 I]epibatidine. Saturation binding assays consistently demonstrated that fewer [125 I]epibatidine binding sites were expressed in IPN and olfactory bulb membranes from $\alpha 2^{-/-}$, compared to $\alpha 2^{+/+}$, mice (Figure 1). Specific binding was saturable, and was well described by a single site Hill fit, in both regions. The amount of specific binding in olfactory bulbs was much less than that in IPN samples, and K_D values for [125 I]epibatidine saturation binding were similar in both regions, as previously demonstrated^[19]. Loss of $\alpha 2$ subunit expression did not significantly alter measured [125 I]epibatidine K_D values in either region. In both regions, $\alpha 2$ -dependent sites were in a minority compared to $\alpha 2$ -independent sites.

[125 I]Epibatidine A85380 inhibition binding, effects of $\alpha 2$ -null mutation Having established that $\alpha 2$ -dependent nAChR populations exist in both mouse IPN and olfactory bulb samples, and could be labeled with [125 I]epibatidine, we next attempted to identify whether these sites were predominantly $\alpha 2\beta 2^*$ or $\alpha 2\beta 4^*$. The sensitivity of IPN and olfactory bulb [125 I]epibatidine binding sites to the $\beta 2^*$ -selective agonist A85380 was tested in [125 I]epibatidine inhibition binding assays (Figure 2). In IPN, $\alpha 2$ -null mutant mice expressed significantly-fewer A85380-resistant (putative $\beta 4^*$) nAChRs than their wild-type counterparts. There was also a strong trend to losing A85380-sensitive (putative $\beta 2^*$) sites following $\alpha 2$ nAChR subunit deletion. In olfactory bulbs samples, $\alpha 2$ nAChR subunit-null mutation resulted in a trend towards

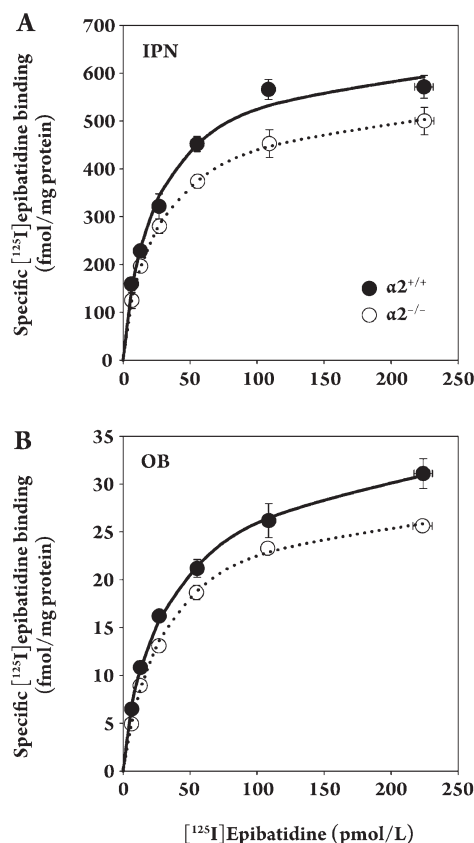


Figure 1. $[^{125}\text{I}]$ Epibatidine saturation filtration binding and effects of $\alpha 2$ -null mutation. $[^{125}\text{I}]$ Epibatidine saturation binding assays were performed on mouse IPN (Panel A) and olfactory bulb (Panel B) membranes. Assays were performed in a 96-well format, using triplicate total and non-specific [1 mmol/L (-)-nicotine added] samples (see Methods for details). Results were compared between preparations from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice, for each region. $n=4$ for each point. In IPN, saturation binding parameters were: $\alpha 2^{+/+}$, $B_{\text{max}}=669\pm 18$ fmol/mg protein, $n_{\text{H}}=1.02\pm 0.14$, $K_{\text{D}}=26.4\pm 1.5$ pmol/L; $\alpha 2^{-/-}$, $B_{\text{max}}=567\pm 8$ fmol/mg protein, $n_{\text{H}}=0.92\pm 0.08$, $K_{\text{D}}=30.9\pm 5.7$ pmol/L. In olfactory bulbs, saturation binding parameters were: $\alpha 2^{+/+}$, $B_{\text{max}}=41.0\pm 1.3$ fmol/mg protein, $n_{\text{H}}=0.79\pm 0.04$, $K_{\text{D}}=45.3\pm 4.6$ pmol/L; $\alpha 2^{-/-}$, $B_{\text{max}}=31.6\pm 2.3$ fmol/mg protein, $n_{\text{H}}=0.98\pm 0.14$, $K_{\text{D}}=40.5\pm 9.3$ pmol/L. For both regions, B_{max} values were significantly reduced following $\alpha 2$ -subunit deletion ($P<0.05$), but n_{H} and K_{D} values were indistinguishable.

lower expression of both A85380-sensitive and -resistant $[^{125}\text{I}]$ epibatidine binding, but neither effect was statistically significant. We wish to emphasize that, in our usage, the terms “A85380-sensitive” and “A85380-resistant” are relative. $[^{125}\text{I}]$ Epibatidine binding at “A85380-resistant,” ($\beta 4^*$) sites is not immune to inhibition by A85380, merely very much less sensitive (approximately 1000-fold^[19]) than that at “A85380-sensitive” ($\beta 2^*$) sites.

Efficacy and potency of $\beta 2$ - and $\beta 4$ -directed mAbs

The A85380 inhibition binding experiments were not able to

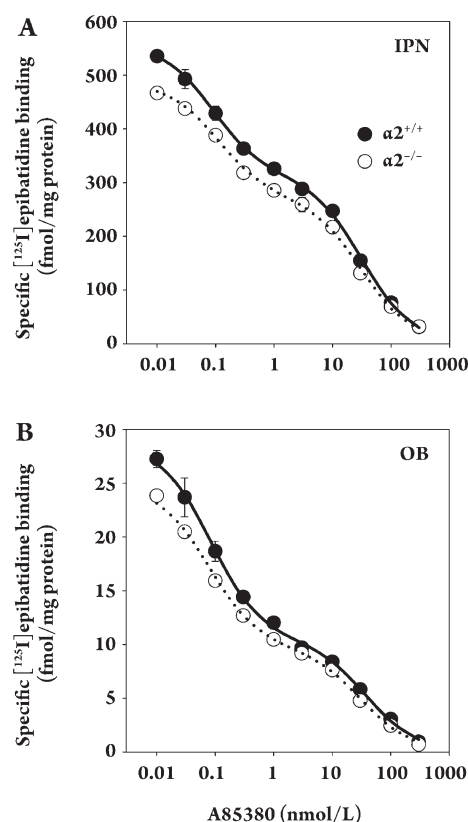


Figure 2. $[^{125}\text{I}]$ Epibatidine A85380 inhibition, filtration binding, effects of $\alpha 2$ -null mutation. Inhibition of $[^{125}\text{I}]$ epibatidine binding by the $\beta 2^*$ nAChR-selective compound A85380 was investigated in mouse IPN (Panel A) and olfactory bulb (Panel B) membranes. Assays were performed in a 96-well format, using triplicate samples. Total binding controls were performed for each assay (with no drug added), as were non-specific determinations [1 mmol/L (-)-nicotine added; see Methods for details]. $n=4$ for each point. For each region, binding was fit to a two-site logistic inhibition model, with B_{max} and IC_{50} values determined for both high-affinity (putative $\beta 2^*$) and low-affinity (putative $\beta 4^*$) sites, for samples from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice. The only significant difference observed was in the density of low-affinity A85380-binding sites in IPN ($\alpha 2^{+/+}$, 313 ± 3 fmol/mg protein; $\alpha 2^{-/-}$, 272 ± 16 fmol/mg protein; $P<0.05$ by Student's t -test).

provide a clear picture of how mouse IPN and olfactory bulb nAChRs were divided between $\alpha 2\beta 2^*$ and $\alpha 2\beta 4^*$ subtypes. In order to further probe the composition of $\alpha 2^*$ nAChRs in these two regions, we employed an immunoprecipitation strategy, using both a $\beta 2$ -directed antibody (mAb 295) and a $\beta 4$ -directed antibody (mAb 337). Titration experiments were first performed to determine the efficacy and potency of these two antibodies against their intended target nAChRs. For mAb 295, the ability of a range of antibody concentrations to immunoprecipitate $[^{125}\text{I}]$ epibatidine (200 pmol/L) binding sites from whole mouse brain was assessed.

For these experiments, $\beta 4^{-/-}$ tissue was used, ensuring that all specific [125 I]epibatidine binding observed would occur at $\beta 2^*$ sites. As shown in Figure 3A, mAb 295 was able to immunoprecipitate > 90 % of the available [125 I]epibatidine binding sites at concentrations >1 μ g/mL. For mAb 337, a

similar set of titration experiments were performed. In the case of this $\beta 4$ subunit-directed mAb, inferior colliculus, IPN, medial habenula, and olfactory bulb tissues were collected from $\beta 2^{-/-}$ mice, and pooled. These regions were chosen since they are known to contain relatively high amounts of

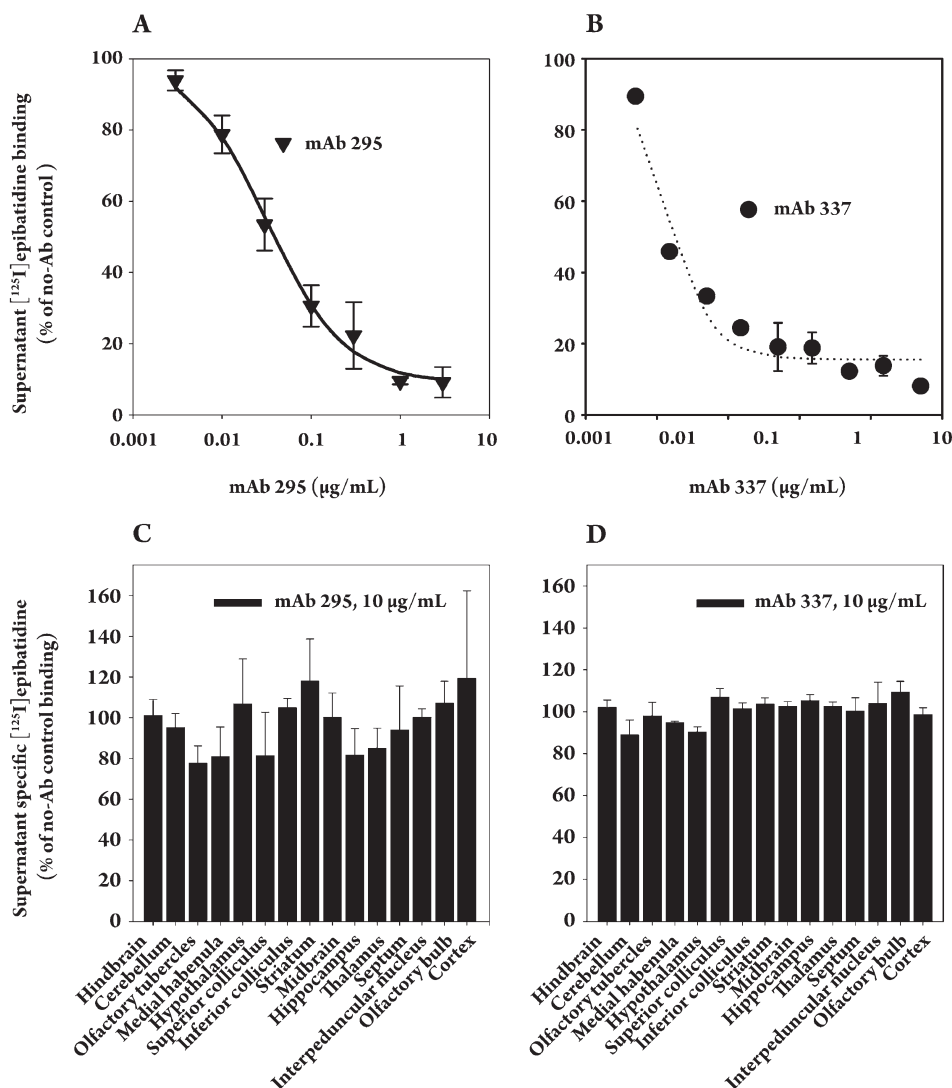


Figure 3. Efficacy, potency, and specificity of $\beta 2$ - and $\beta 4$ -directed mAbs. Triton X-100 (2 %) extracted nAChR-antibody complexes were captured on protein G CPG magnetic beads. Supernatant receptors were recovered using PEG-8000 (20% w/v). nAChR contents were determined for both bead-bound and supernatant fractions using [125 I]epibatidine (200 pmol/L) in a filtration binding format. $n=4$ for each point. Panel A). For mAb 295, potency and efficacy were determined by titration, using whole-brain extracts from $\beta 4$ subunit-null mice. Panel B). For mAb 337, potency and efficacy were determined by titration, using extracts from pooled inferior colliculus, IPN, medial habenula, and olfactory bulb regions of $\beta 4$ subunit-null mice. Panel C). Specificity of mAb 295 (10 μ g/mL; maximally effective concentration) immunoprecipitation was determined in triton extracts from fifteen $\beta 2^{-/-}$ mouse brain regions, which together represent the whole brain. As determined by one-way ANOVA, no significant immunoprecipitation of [125 I]epibatidine binding sites was seen in any region in the absence of the target $\beta 2$ subunit, demonstrating the specificity of mAb 295 immunoprecipitation under these conditions. Panel D). Specificity of mAb 337 (10 μ g/mL; maximally effective concentration) immunoprecipitation was determined in Triton extracts from the same fifteen mouse brain regions, this time from $\beta 4^{-/-}$ mice. Again, as determined by one-way ANOVA, no significant immunoprecipitation of [125 I]epibatidine binding sites was seen in any region in the absence of the target $\beta 4$ subunit, demonstrating the specificity of mAb 295 immunoprecipitation under these conditions.

$\beta 4^*$ nAChRs^[22]. $\beta 2^{-/-}$ tissue was used to ensure that, for this set of experiments, all of the specific [¹²⁵I]epibatidine binding observed would occur at $\beta 4^*$ sites. mAb 337 was highly potent, producing maximal immunoprecipitation at concentrations $>0.3 \mu\text{g/mL}$ (Figure 3B). However, it was not quite as efficacious as mAb 295, precipitating about 80% of the sites available to it. This observation mirrors similar results obtained for mAb 337 when it was first characterized^[23].

Specificity of $\beta 2$ - and $\beta 4$ -directed mAbs On the basis of the titration experiments, maximally-effective concentrations of mAbs 295 and 337 were chosen ($10 \mu\text{g/mL}$ for both). The specificity of each antibody at the chosen concentration was assessed by performing immunoprecipitation reactions in fifteen regions across the mouse brain, using tissue from mice lacking expression of the target subunit

(*ie*, $\beta 2^{-/-}$ tissue for mAb 295, $\beta 4^{-/-}$ tissue for mAb 337). Together, these regions represent the whole mouse brain. As shown in Figure 3, Panels C and D, no significant immunoprecipitation was observed in any region lacking the target subunit, for either antibody. This strongly suggests that both antibodies are specific for their intended targets when used in the immunoprecipitation protocols described here.

$\beta 2$ -immunoprecipitation, effects of $\alpha 2$ -null mutation

Using mAb 295, $\beta 2^*$ nAChRs were collected from detergent extracts of IPN and olfactory bulb membranes. Both captured and supernatant nAChRs were quantified using [¹²⁵I]epibatidine (200 pmol/L) in a filtration binding format. In both regions, the great majority of nAChRs captured by mAb 295 were A85380-sensitive, as would be expected for $\beta 2^*$ nAChRs (Figure 4). However, particularly in IPN,

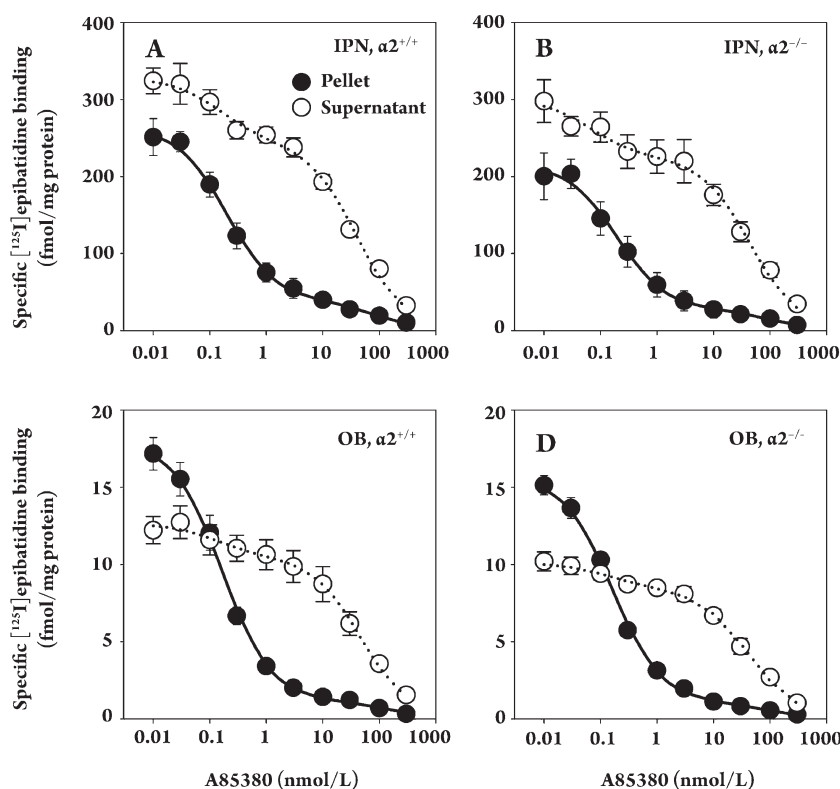


Figure 4. $\beta 2$ -immunoprecipitation and effects of $\alpha 2$ -null mutation. Triton X-100 (2%) extracted nAChR-mAb 295 ($10 \mu\text{g/mL}$) complexes were captured on protein G CPG magnetic beads. Supernatant receptors were collected as before. Both captured and supernatant nAChRs were quantified using [¹²⁵I]epibatidine (200 pmol/L) in a filtration binding format, and inhibition of [¹²⁵I]epibatidine binding by the $\beta 2^*$ nAChR-selective compound A85380 was determined. $n=4$ for each point. Panels A and B: IPN extracts from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice, respectively. Panels C and D: olfactory bulb extracts from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice, respectively. For each region, and for both immunoprecipitated ($\beta 2^*$ nAChRs, solid points) and supernatant (predominantly non- $\beta 2^*$ nAChRs, hollow points) binding was fit to a two-site logistic inhibition model, with B_{max} and IC_{50} values determined for both high-affinity and low-affinity A85380-binding sites, for samples from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice. In IPN, a significant difference was observed in the density of high-affinity A85380-binding sites immunoprecipitated by the $\beta 2$ -specific mAb 295 ($\alpha 2^{+/+}$, $B_{\text{max}}=224 \pm 11 \text{ fmol/mg protein}$; $\alpha 2^{-/-}$, $B_{\text{max}}=165 \pm 19 \text{ fmol/mg protein}$; $P<0.05$ by Student's *t*-test). In OB, the IC_{50} of A85380 inhibition at low A85380-affinity, supernatant (predominantly non- $\beta 2^*$) nAChRs was significantly affected in the absence of $\alpha 2$ expression ($\alpha 2^{+/+}$, $\text{IC}_{50}=142 \pm 32 \text{ nmol/L}$; $\alpha 2^{-/-}$, $\text{IC}_{50}=43.1 \pm 32 \text{ nmol/L}$; $P<0.05$ by Student's *t*-test). No other assay parameters were significantly affected by $\alpha 2$ genotype.

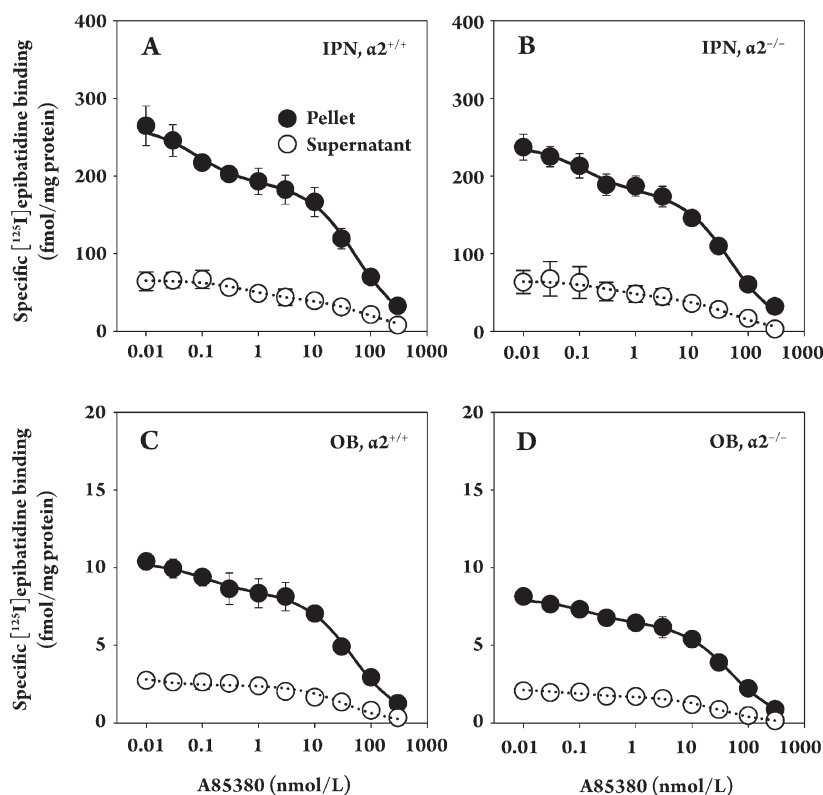


Figure 5. $\beta 4$ -immunoprecipitation in $\beta 2$ -depleted samples and effects of $\alpha 2$ -null mutation. $\beta 2^*$ nAChR-depleted supernatants from the mAb 295 immunoprecipitations were subjected to a second round of immunoprecipitation with mAb 337 (10 $\mu\text{g}/\text{mL}$). Complexes were again captured on protein G CPG magnetic beads. Supernatant receptors were collected as before. In line with the results in Figure 3D and ref [23], mAb 337 immunoprecipitation was incompletely efficacious. Both captured and supernatant nAChRs were quantified using [^{125}I]epibatidine (200 pmol/L) in a filtration binding format, and inhibition of [^{125}I]epibatidine binding by the $\beta 2^*$ nAChR-selective compound A85380 was determined. $n=4$ for each point. Panels A and B: IPN extracts from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice, respectively. Panels C and D: olfactory bulb extracts from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice, respectively. For each region, and for both immunoprecipitated ($\beta 4^*$ nAChRs, solid points) and supernatant (hollow points) binding was fit to a two-site logistic inhibition model, with B_{max} and IC_{50} values determined for both high-affinity and low-affinity A85380-binding sites, for samples from $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ mice. In IPN, no significant differences in binding parameters were seen between $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ samples. In OB, the density of low A85380-affinity, immunoprecipitated ($\beta 4^*$) nAChRs was significantly lower in the absence of $\alpha 2$ expression ($\alpha 2^{-/-}$, $B_{\text{max}}=10.3\pm 0.6$ fmol/mg protein; $\alpha 2^{+/+}$, $B_{\text{max}}=7.99\pm 0.47$ fmol/mg protein; $P<0.05$ by Student's t -test). No other assay parameters were significantly affected by $\alpha 2$ genotype.

a small proportion of the binding sites precipitated using mAb 295 were A85380-resistant. Given that earlier experiments had demonstrated the specificity of mAb 295 for $\beta 2^*$ nAChRs, it is unlikely that these sites were captured non-specifically. Instead, they may represent a small proportion of nAChRs that contain both $\beta 2$ and $\beta 4$ subunits in the same complex. In IPN, $\beta 2^*$ nAChR expression was significantly reduced by $\alpha 2$ -null mutation (Figure 4, panels A, B). No other significant effects were seen on the sizes, or A85380 sensitivities, of the nAChR populations in IPN. However, in the olfactory bulb from nAChR $\alpha 2$ subunit-null mice, there was no significant effect on the expression of A85380-sensitive or -resistant nAChRs, either in the precipitated or supernatant populations. On the other hand, the IC_{50} for A85380 inhibition of low A85380 affinity, supernatant (pre-

dominantly non- $\beta 2^*$) nAChRs from the olfactory bulb was significantly affected in the absence of $\alpha 2$ subunit expression.

$\beta 4$ -immunoprecipitation in $\beta 2$ -depleted samples, effects of $\alpha 2$ -null mutation The supernatant samples (now depleted of $\beta 2^*$ nAChRs) from the preceding experiment were next used in $\beta 4$ immunoprecipitation experiments. In line with mAb 337's less-than-complete efficacy, about 75% of nAChRs were captured (Figure 5). The remaining nAChRs had similar proportions of A85380-sensitive and resistant binding sites to those that were captured, suggesting that there was no systematic difference between the nAChRs that were captured, and the rest. In IPN samples, no significant differences were seen between $\alpha 2^{+/+}$ and $\alpha 2^{-/-}$ samples, in terms of the affinities or numbers of A85380-sensitive and -resistant [^{125}I]epibatidine binding sites (Figure 5A, 5B).

However, In OB, the A85380-resistant population immunoprecipitated by mAb 337 was significantly reduced by $\alpha 2$ -null mutation (Figure 5C, 5D).

Discussion

Saturation binding assays illustrated a modest, but measurable, $\alpha 2$ dependence for high-affinity [125 I]epibatidine binding in both IPN and olfactory bulbs of mice. This finding is compatible with previous mRNA *in situ* hybridization studies that indicated a particularly high concentration of $\alpha 2$ mRNA expression in these two nuclei^[7, 8]. Although $\alpha 2^*$ nAChRs are a minority population within both of these nuclei, their expression within extremely tightly-defined subregions (dorsal IPN, internal plexiform layer of the olfactory bulbs) means that $\alpha 2$ nAChRs may play significant roles, despite their low overall expression levels. Certainly, recent evidence that hippocampal $\alpha 2^*$ nAChRs can powerfully modulate nicotine's effects on long-term potentiation in the hippocampal CA1 region^[17] demonstrates that numerically under-represented nAChR populations can have important physiological effects.

Even within the IPN and olfactory bulbs, expression levels were low enough to make ligand binding assays difficult. Reflecting these difficulties, [125 I]epibatidine inhibition binding assays using the $\beta 2^*$ nAChR subtype-selective agonist A85380 were inconclusive, perhaps suggesting complex association of $\alpha 2$ with both $\beta 2$ and $\beta 4$ subunits in the mouse IPN and olfactory bulb. The problems with the assays using IPN and olfactory bulb preparations suggest that investigations of $\alpha 2^*$ populations in other mouse brain regions will require alternative approaches. In particular, studies in mouse hippocampus would be of interest, given the recently-discovered role of $\alpha 2^*$ nAChRs in modulating LTP in murine CA1^[17].

The association of $\alpha 2$ nAChR subunits with $\beta 2$ and $\beta 4$ subunits was further investigated using an immunoprecipitation strategy. Preliminary experiments were performed to ascertain the potency, efficacy, and specificity of mAbs 295 (*vs* $\beta 2$ subunits) and 337 (*vs* $\beta 4$ subunits). These pilot experiments used $\beta 2$ and $\beta 4$ subunit-null tissue as negative controls, similar to several previous studies^[20, 24, 25], further extending the application of this useful approach. Both mAb 295 and 337 showed high potency and complete specificity for their targets ($\beta 2$ and $\beta 4$ nAChR subunits, respectively). Interestingly, although both mAbs were highly efficacious, mAb 337 immunoprecipitation was consistently slightly incomplete even at very high antibody concentrations. This result is similar to that reported when mAb 337 was first characterized^[26]. The original explanation for this observa-

tion was that a small proportion of the $\beta 4$ epitope recognized by mAb 337 is likely in either "the wrong conformation, obscured by another protein, or is consistently proteolyzed." Whichever is the correct explanation, a similar factor appears to be present in this set of (mouse brain) studies as in the (cell line) studies previously described.

The immunoprecipitation experiments allowed for the $\beta 2^*$ and $\beta 4^*$ nAChR populations to be effectively separated from each other before pharmacological analysis was applied. This allowed more-definitive conclusions to be drawn regarding the β -subunit composition of $\alpha 2^*$ nAChRs in mouse IPN and olfactory bulbs. In the IPN, $\alpha 2$ subunit-null mutation was predominantly associated with changes in nAChRs precipitated by mAb 295 (against the $\beta 2$ subunit). In particular, A85380-sensitive sites captured by mAb 295 were significantly reduced in $\alpha 2^{-/-}$ IPN samples, compared to wild-type controls. In contrast, $\alpha 2$ deletion is associated with changed A85380 binding affinity in olfactory bulb, non- $\beta 2^*$ nAChRs (not precipitated by mAb 295). Furthermore, fewer olfactory bulb nAChRs were able to be precipitated by mAb 337 (against the $\beta 4$ subunit) in $\alpha 2^{-/-}$ samples, compared to wild-type controls. It also appears that the IPN, in particular, expresses a substantial proportion of $\beta 4\beta 2^*$ nAChRs, given the quite substantial proportion of A85380-resistant [125 I]epibatidine binding sites captured by mAb 295 immunoprecipitation in this region. Together these findings imply that, in IPN, $\alpha 2$ predominantly, but not exclusively, partners with $\beta 2$ subunits. In OB, however, $\alpha 2$ subunits are largely partnered with $\beta 4$ nAChR subunits. The current IPN observation broadly agrees with the very recent findings of Grady *et al*, who also noted that IPN $\alpha 2^*$ nAChRs were of the $\alpha 2\beta 2^*$ subtype^[12]. Interestingly, these authors did not identify any $\alpha 2$ component in the IPN $\beta 4^*$ nAChR complement, while the present study did suggest a small amount of $\alpha 2\beta 2\beta 4^*$ expression may occur in IPN. This slight discrepancy may reflect the relative efficacies of the antibodies and immunoprecipitation protocols employed in the two studies. In any case, the general finding that at least a large majority of IPN $\alpha 2$ nAChR subunits are partnered with $\beta 2^*$ subunits is confirmed.

In conclusion, this study demonstrates that mouse brain $\alpha 2$ nAChRs are sufficiently prevalent in mouse IPN and olfactory bulb to be detected with ligand binding and immunoprecipitation approaches. In the IPN, $\alpha 2\beta 2^*$ nAChRs are likely the predominant subtype of $\alpha 2$ nAChRs, although some may also contain $\beta 4$ subunits in the same receptor complex. In the olfactory bulbs, $\alpha 2$ nAChR subunits predominantly assemble into $\alpha 2\beta 4^*$ nAChRs. These findings indicate that $\alpha 2^*$ nAChR pharmacology is likely to be com-

plex, a finding that may have particularly important implications if, as may be the case, $\alpha 2^*$ nAChRs are more prevalent in primate (including human) than rodent brain^[13].

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Author contribution

Paul WHITEAKER designed experiments, wrote the paper, and performed and directed research; Jennifer A WILKING, Robert WB BROWN and Robert J BRENNAN performed research; Allan C COLLINS assisted with experimental design; Jon M LINDSTROM contributed reagents and assisted with experimental design; Jim BOULTER contributed subunit-null mice and assisted with experimental design.

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