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## **$\beta$ 3 Subunits Promote Expression and Nicotine-Induced Upregulation of Human Nicotinic $\alpha$ 6\* AChRs Expressed in Transfected Cell Lines**

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**ABBREVIATIONS:** AChR, acetylcholine receptor;  $\alpha$ Bgt,  $\alpha$  bungarotoxin; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; MIR, main immunogenic region; mAb, monoclonal antibody; PBS, phosphate buffered saline; TPBS, 0.5% Triton X-100 in PBS

## ABSTRACT

Nicotinic acetylcholine receptors (AChRs) containing  $\alpha 6$  subunits are typically found at aminergic nerve endings where they play important roles in nicotine addiction and Parkinson's disease.  $\alpha 6^*$  AChRs usually contain  $\beta 3$  subunits.  $\beta 3$  subunits are presumed to assemble only in the accessory subunit position within AChRs where they do not participate in forming ACh binding sites. Assembly of subunits in the accessory position may be a critical final step in assembly of mature AChRs. Human  $\alpha 6$  AChRs subtypes were permanently transfected into human tsA201 HEK cell lines.  $\alpha 6\beta 2\beta 3$  and  $\alpha 6\beta 4\beta 3$  cell lines were found to express much larger amounts of AChRs and were more sensitive to nicotine-induced increase in the amount of AChRs than were  $\alpha 6\beta 2$  or  $\alpha 6\beta 4$  cell lines. The increased sensitivity to nicotine-induced upregulation was not due to a  $\beta 3$  induced increase in affinity for nicotine, but probably due to a direct effect on assembly of AChR subunits. HEK cells express only a small amount of mature  $\alpha 6\beta 2$  AChRs, but many of these are on the cell surface. This contrasts with *Xenopus* oocytes which express a large amount of incorrectly assembled  $\alpha 6\beta 2$  subunits which bind cholinergic ligands but form large amorphous intracellular aggregates. mAbs were made to the  $\alpha 6$  and  $\beta 3$  subunits to aid in the characterization of these AChRs. The  $\alpha 6$  mAbs bind to epitopes C-terminal of the extracellular domain. These data demonstrate that both cell type and the accessory subunit  $\beta 3$  can play important roles in  $\alpha 6^*$  AChR expression, stability, and upregulation by nicotine.

Nicotinic acetylcholine receptors (AChRs) are composed of five homologous subunits (Sine and Engel, 2006; Lindstrom, 2000). Heteromeric neuronal AChRs contain two ACh binding sites formed by the interfaces of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ , or  $\alpha 6$  subunits with  $\beta 2$  or  $\beta 4$  subunits. The fifth “accessory” subunit does not participate in forming an ACh binding site but contributes to the channel lining and influences assembly, transport, and function of AChRs. The accessory position is often occupied by  $\beta 2$  or  $\beta 4$  subunits, but  $\alpha 4$  can also assemble there (Kuryatov et al., 2005).  $\beta 3$  and  $\alpha 5$  can assemble only in this position.

AChRs containing  $\alpha 6$  subunits ( $\alpha 6^*$  AChRs) comprise minor subtypes selectively localized in the endings of aminergic neurons (Zoli et al., 2002; Champtiaux et al., 2003; Gotti et al., 2005b).  $\alpha 6^*$  AChRs contribute to nicotine-stimulated dopamine release from striatal synaptosomes (Azam et al., 2005), are selectively lost in animal models of Parkinson’s disease, and are potential targets for Parkinson’s disease therapy (Quik and McIntosh, 2006).  $\alpha 6^*$  AChRs are the major non- $\alpha 4^*$  AChR expressed in the optic tract (Gotti et al., 2005b).  $\beta 3$  subunits are usually found in  $\alpha 6^*$  AChRs, and knockout of  $\beta 3$  reduces but does not eliminate expression of  $\alpha 6^*$  AChRs (Gotti et al., 2005a).

Nicotine upregulates the amount of brain AChRs (Flores et al., 1992). Nicotine treatment has been reported to both increase (Parker et al., 2004) and decrease the amount of brain  $\alpha 6^*$  AChRs (Lai et al., 2005; McCallum et al., 2006; Mugnaini et al., 2006). In transfected HEK cell lines, nicotine applied overnight increases the amount of human  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  AChRs, but not  $\alpha 3\beta 4$  or  $\alpha 4\beta 4$  AChRs, primarily by acting as a pharmacological chaperone to promote assembly of AChRs (Wang et al., 1998; Salette et al., 2005; Kuryatov et al., 2005; Corringer et al., 2006).

Putative assembly intermediates the size of  $\alpha 4\beta 2\alpha 4\beta 2$  tetramers have been identified which could assemble with accessory subunits in a final step to produce mature AChRs (Kuryatov et al., 2005). Nicotine also contributes to upregulation by increasing the lifetime of surface membrane AChRs (Kuryatov et al., 2005). Nicotine applied to transfected HEK cell lines for five days upregulated rat AChRs containing  $\alpha 2$ ,  $\alpha 3$ , or  $\alpha 4$  in combination with  $\beta 2$  or  $\beta 4$  subunits (Xiao and Kellar, 2004).

It has been challenging to express  $\alpha 6$  AChRs (Gerzanich et al., 1997). In *Xenopus* oocytes, human  $\alpha 6\beta 4$  AChRs were functional and  $\alpha 6\beta 4\beta 3$  AChRs were expressed at a higher level (Kuryatov et al., 2000). Although co-expression of  $\alpha 6$  and  $\beta 2$  produced abundant ACh binding sites, they were on amorphous aggregates within the oocytes. Chimeric subunits with the extracellular domain of  $\alpha 6$  and the remainder of either  $\alpha 3$  or  $\alpha 4$  subunits assembled efficiently with either  $\beta 2$  or  $\beta 4$  subunits in oocytes (Kuryatov et al., 2000). In human BOSC 23 cells, chicken  $\alpha 6\beta 2$  were AChRs expressed at a lower level than  $\alpha 4\beta 4$  (Fucile et al., 1998). Attempts to express human  $\alpha 6\beta 2$ ,  $\alpha 6\beta 2\beta 3$ ,  $\alpha 6\beta 4$ , and  $\alpha 6\beta 4\beta 3$  AChRs in transfected SH-EPI cell lines were unsuccessful, but the  $\alpha 6\beta 4\beta 3\alpha 5$  subunit combination exhibited cholinergic ligand binding (Grinevich et al., 2005). Chimeric subunits with the extracellular domain of  $\alpha 6$  and the remainder of  $\alpha 4$  formed functional AChRs in HEK cell lines (Evans et al., 2003).  $\alpha 6$  subunits are closely related in sequence to  $\alpha 3$  subunits, and  $\beta 3$  subunits are closely related to  $\alpha 5$  subunits (Lindstrom, 2000; Le Novere et al., 2002). Permanently transfected HEK tsA201 cells have been used to express human  $\alpha 3^*$  and  $\alpha 4^*$  AChRs (Wang et al., 1998; Nelson et al., 2001; Kuryatov et al., 2005).  $\alpha 3\beta 2$  AChRs assembled efficiently in *Xenopus* oocytes (Wang et al.,

1996; Gerzanich et al., 1998), but in HEK cells they do not assemble efficiently unless upregulation is induced by nicotine or culture at low temperatures (Wang et al., 1998). Co-expression in HEK cells of  $\alpha 3\beta 2$  with  $\alpha 5$  increases expression, but co-expression of  $\alpha 3\beta 4$  with  $\alpha 5$  somewhat decreases expression (Wang et al., 1998). In oocytes,  $\alpha 5$  subunits alter desensitization, pharmacology, and  $\text{Ca}^{++}$  permeability of  $\alpha 3$  AChRs (Gerzanich et al., 1998), so it might be expected that  $\beta 3$  subunits would similarly have substantial effects on  $\alpha 6$  AChRs.

Here we report the production of stably transfected tsA201 HEK lines expressing four subtypes of human  $\alpha 6^*$  AChRs and their initial characterization. Expression of  $\beta 3$  with either  $\alpha 6\beta 2$  or  $\alpha 6\beta 4$  subunit combinations increased the amount of AChRs expressed and increased their sensitivity to upregulation by nicotine. mAbs were made to human  $\alpha 6$  and  $\beta 3$  subunits to aid in the characterization of these AChRs.

## MATERIALS AND METHODS

**cDNAs:** Human  $\alpha 6$ ,  $\beta 2$ , and  $\beta 4$  cDNAs were cloned in this lab as previously described (Anand and Lindstrom, 1990; Gerzanich et al., 1997; Kuryatov et al. 2000). Human  $\beta 3$  was obtained from Christopher Grantham at the Janssen Research Foundation (Belgium) and subcloned into pcDNA 3.1/Zeo(+) for transfection.  $\alpha 6$  was subcloned into pEF6/Blasticidin(+).  $\beta 2$  and  $\beta 4$  were separately cloned into pRc-CMV/Geneticin(+). All vectors were obtained from Invitrogen Life Technologies.

**Cell Line Culture and Transfection:** Human embryonic kidney tsA201 cell lines

(Margolskee et al., 1993) were maintained in Dulbecco Modified Eagle Medium (DMEM, high glucose) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 2 mM L-glutamine at 37° C, 5% CO<sub>2</sub> at saturating humidity in a water jacket incubator. HEK cells were transfected using the Fugene 6 DNA Transfection Kit, according to the manufacturer's instructions, with human  $\alpha 6$  (pEF6/Blasticidin), human  $\beta 2$  (pRc-CMV/Geneticin(+)), or human  $\beta 4$ (pRc-CMV/Geneticin(+)).  $\alpha 6\beta 2$  was created by transfecting HEK cells with  $\alpha 6$  and  $\beta 2$  cDNAs.  $\alpha 6\beta 4$  was created by transfecting HEK cells with  $\alpha 6$  and  $\beta 4$  cDNAs. Selective pressure for cells containing  $\alpha 6$  was applied with Blasticidin (5 $\mu\text{g/ml}$ ) starting 72 hours after transfection.  $\beta 2$  and  $\beta 4$  cell lines were similarly selected with Geneticin (600  $\mu\text{g/ml}$ ).  $\alpha 6\beta 2$  and  $\alpha 6\beta 4$  AChRs were assayed for expression and screened by [<sup>3</sup>H] epibatidine binding. Stably transfected  $\alpha 6\beta 4$  and  $\alpha 6\beta 2$  lines were co-transfected with human  $\beta 3$  (pcDNA 3.1/Zeocin(+)) to produce  $\alpha 6\beta 4\beta 3$  and  $\alpha 6\beta 2\beta 3$  AChRs. Selective pressure for cell lines containing  $\beta 3$  was applied using Zeocin (500  $\mu\text{g/ml}$ ) starting 72 hours after transfection. AChRs containing  $\beta 3$  were selected for high expression based on liquid phase radioimmune assays.  $\beta 3$  transfected cell lines were plated in serial dilution on Costar 96 well plates. Surviving colonies were plated individually to Costar 35mm culture plates and then grown to confluency. Cells were detached with ice cold DMEM and extracted as described above into a 1.5 ml microfuge tube. AChRs were incubated with 2.5 nM [<sup>3</sup>H] epibatidine and 5  $\mu\text{l}$  of  $\beta 3$  antiserum, acquired from Santa Cruz Biotechnologies (Santa Cruz, CA), for 1-2 hours at room temperature. AChRs were precipitated by secondary antibody

incubation with rabbit anti-goat antiserum for 1-2 hours at room temperature and then pelleted at 13,000x g for 10 minutes. Pellets were washed three times with 1 ml of 0.5% Triton X-100 in PBS +10mM NaN<sub>3</sub> to remove excess radioligand. Washing solution was aspirated from the pellet before adding 50 µl of 0.1 N NaOH to solubilize the pellet. One ml of Optiphase 'SuperMix' (PerkinElmer Life Sciences) was added to each sample. Samples with scintillation fluid were set on rotation for 1 hour at room temperature before counting in a 1450 Microbeta Trilux Liquid Scintillation & Luminescence Counter (Perkin-Elmer Life Sciences).

**Membrane Fraction [<sup>3</sup>H] Epibatidine Binding:** To assess relative expression levels across α6 containing AChR types expressed in HEK cells, stably transfected HEK cells were grown to confluency on Costar 35 mm tissue culture plates (approximately 1x 10<sup>6</sup> cells/plate) and then detached by cold DMEM and collected by centrifugation at 500x g. Cells were washed once with 1 ml buffer A (50 mM NaPO<sub>4</sub> pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide, 2 mM phenylmethylsulfonylflouride), pelleted by centrifugation at 13,000x g and then resuspended in buffer A. Cells in buffer A were incubated with 2 nM [<sup>3</sup>H] epibatidine for 30 minutes at room temperature with agitation. Cell membrane fractions were washed three times with PBS +10 mM NaN<sub>3</sub> on Whatman glass filters (GF/F) pretreated with 1% polyethylenimine for 1 hour and dried on blotting paper. Filters containing AChRs bound to radioligands were counted in 1.5 ml microfuge tubes using 1ml of Optiphase SuperMix scintillation fluid in a 1450 Microbeta Trilux liquid scintillation counter (Perkin-Elmer Life Sciences). Results were standardized to the wet weight of the cells after the first wash in buffer A.



**Previously Used Antisera and mAbs:** A rat antiserum to bacterially-expressed  $\alpha 6$  subunit sequences (excluding the transmembrane domains) was raised as described by Kuryatov et al. (2000). The rat mAb 210 binds to the main immunogenic region on native  $\alpha 1$ ,  $\alpha 3$  and  $\alpha 5$  (Lindstrom, 2000) and  $\beta 3$  (unpublished data). The rat mAb 295 binds to the extracellular surface of AChR  $\beta 2$  subunits when they are assembled with  $\alpha$  subunits (Lindstrom, 2000; Kuryatov et al., 2005). The mouse mAb 337 was raised to bacterially-expressed human  $\beta 4$  subunit large cytoplasmic domain (Nelson et al., 2001).

**Preparation of New mAbs:** Human  $\alpha 6$  and  $\beta 3$  subunits lacking the transmembrane domains of these subunits were constructed in the pET-26b(+) vector (Novagen) and expressed in bacteria.  $\alpha 6$  subunits were expressed in oocytes and extracted with 2% Triton X-100 as described in Kuryatov et al. (2000). The extracts were incubated with mAb 295 coupled to CH Sepharose (Pharmacia) for purifying AChRs for use in immunization or with cell culture supernatants in protein A-coated microtiter wells for solid phase RIA.

Female BALB/c mice, 3-4 weeks of age, were obtained from Charles River Laboratories. All animals were handled in accordance with guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania under an approved protocol on file with that office. IACUC operates under an institutional Animal Welfare Assurance (A3079-01) on file with the Office for Protection from Research Risks at the National Institutes of Health.

BALB/c mice were immunized, and then boosted at three-week intervals with 40  $\mu$ g per mouse of bacterially-expressed subunit constructs lacking the transmembrane domains of the subunits in TiterMax (TiterMax USA, Inc.). The titers were monitored by test bleeds against

corresponding subunits in ELISA assay. Five days after a final boost with antigen, the harvested splenic lymphocytes ( $\sim 10^8$ ) from the animal with the highest titers were fused with SP2/0 myeloma cells ( $1 \times 10^8$ ) using 50% polyethylene glycol (Kodak 1450) and inoculated into 24 96-well plates (Costar 3595).

Initial screening was done by ELISA assay as previously described (Kuryatov, et al., 2005). To eliminate mAbs which crossreacted with closely related subunits, the crossreaction of mAbs with a subunit which has the highest homology with the immunogen was tested ( $\alpha 3$  in the case of  $\alpha 6$  and  $\alpha 5$  in the case of  $\beta 3$ ). mAbs which could bind both denatured subunits and native AChRs, were selected using [ $^3\text{H}$ ] epibatidine-labelled native AChRs in RIAs, and then cloned by a limiting dilution method.

To determine titers of antibody to native AChRs, various concentrations of mAbs were incubated in 100  $\mu\text{l}$  of buffer C containing a 0.5 nM concentration of AChRs and 1.5 nM [ $^3\text{H}$ ] epibatidine. After overnight at 4°C, 25  $\mu\text{l}$  of Zysorbin (Zymed Laboratories) were added into each tube, followed by a 30-min incubation at room temperature. The material in the tubes was diluted into 1 ml with 0.5 % Triton X-100 in PBS (TPBS). After a 5-min centrifugation at 4,000x g, the supernatant was removed by aspiration. The pellet was washed with 1 ml of TPBS and then suspended in 100  $\mu\text{l}$  of 0.1N NaOH. The suspension was mixed with 900  $\mu\text{l}$  of Optiphase "SuperMix" scintillation fluid (Fisher Scientific). The amount of bound [ $^3\text{H}$ ] epibatidine was determined by liquid scintillation counting for 5 min. Background was determined by substituting normal mouse serum for the mAb.

To determine cross reactivity of mAbs to related subunits with dot blot immunoassay, 167 ng/well of various bacterially-expressed human AChR subunits were gravity-blotted onto

prewetted nitrocellulose membranes in 100  $\mu$ l of TPBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) using a Bio-Dot apparatus (Bio-Rad). 300  $\mu$ l of TPBS containing 1% bovine serum albumin was applied into each well to block nonspecific-antibody binding in subsequent steps. After blocking, the membranes were incubated for 1 hr with a 1 $\mu$ g/ml of mAb or a 1:500 dilution of rat antiserum specific for the different subunits as controls. The membranes were rinsed three times with TPBS containing 0.05% Tween 20 and then probed for 1 hr with a 1:2000 dilution of biotinylated goat anti-mouse IgG (Kirkegaard & Perry Laboratories), followed by a 1 hour incubation with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories), and a final incubation in peroxidase substrate to visualize antibody binding. A 1:2000 dilution of biotinylated goat anti-rat IgG (Kirkegaard & Perry Laboratories) was used under conditions where rat antiserum was used as the first antibody instead of a mAb. Membranes were washed five times with TPBS containing 0.05% Tween-20 and then two times with TPBS before substrate addition.

**AChR Extraction from HEK Cells with Triton X-100:** HEK cells expressing AChRs were grown in Costar 10 cm or 15 cm plates and then upregulated by exposure to 100  $\mu$ M nicotine overnight at 37°C. Cells were detached using 5 or 10 ml of ice cold DMEM and then collected via centrifugation at 500x g. DMEM was aspirated from the pellet. Cells were suspended with 1 ml of buffer A, then transferred to a 1.5 ml Eppendorf microfuge tube and collected via centrifugation at 13,000x g for 15 minutes at 4° C. Buffer A was aspirated from the pellets, which were then weighed. AChRs were extracted with three volumes of the pellet weight using buffer C (buffer A with 2% Triton X-100). The suspension was gently rotated for 1 hour at room temperature. Cell debris was pelleted by centrifugation at 13,000x g for 15 minutes

at 4° C. Supernatant containing AChRs in Triton X-100 was removed to new 1.5 ml centrifuge tubes and used immediately, keeping samples at 0-4° C.

**Western Blots:** AChRs were extracted with 2% Triton X-100 as described above from either HEK cell lines or *Xenopus* oocytes expressing either  $\alpha 6\beta 2$ , chimeric  $\alpha 6/\alpha 3 \beta 2$ , or chimeric  $\alpha 3/\alpha 6 \beta 2$  AChRs. Samples were electrophoresed in pre-cast Novex 10% polyacrylamide tris-glycine gels (San Diego, CA) under reducing conditions. Western transfer was done using a semi-dry electroblotting chamber (Semi-Phor, Hoefer Scientific Instruments, San Francisco, CA) to Trans-Blot® Medium PVDF membrane (Bio-Rad, Hercules, CA). Blots were quenched with 5% dried milk (Carnation) in 0.5% Triton X-100 in PBS, 10 mM  $\text{NaN}_3$ . mAbs were used as indicated at 1:1000 dilution in milk blocking solution. Blots were probed overnight at 4° C on a shaker followed by three washes with 0.5% Triton X-100 in PBS, 10 mM  $\text{NaN}_3$ . Blots were then incubated with 2 nM [ $^{125}\text{I}$ ] goat anti-mouse IgG (specific activity  $2.5 \times 10^{18}$  cpm/mol) overnight on a shaker at 4° C followed by three washes with TPBS. Autoradiography was done at -80° C with Kodak Biomax film using a Kodak MS screen for the indicated time periods.

**Agonist Binding on Fixed Cells:** Cells were plated onto Costar 96 well white with clear bottom plates and grown to 70% confluency. Nicotine was added to induce upregulation of AChRs. Cells were incubated overnight at 37° C. Cells grown to confluency were exposed to one volume of 10% formalin added directly to the growth medium for 1 hour at room temperature in order to fix the cells to the wells. Cells were washed free of agonist and formalin with 200 $\mu\text{l}$  of PBS + 10 mM  $\text{NaN}_3$  three times and stored at 4°C with one volume of PBS-  $\text{NaN}_3$  until use. Agonists were applied at indicated concentrations with [ $^3\text{H}$ ] epibatidine at 2 nM.

Binding was conducted at room temperature for 2 hours with gentle agitation. Plates were again washed to remove unbound ligands as above. [<sup>3</sup>H] epibatidine was eluted from the AChRs by 50  $\mu$ l of 0.1 N NaOH. 200  $\mu$ l of Optiphase 'SuperMix' (PerkinElmer Life Sciences) scintillation fluid were added to each well. Plates were then shaken at room temperature for 1-2 hours before scintillation counting. Comparison of the maximum number of epibatidine binding sites in all four cell lines after upregulation with nicotine revealed that in fixed cells 93 $\pm$ 7% of the binding sites observed in membrane fragments were detected on fixed cells. Binding to fixed cells was faster and easier than using membrane fragments and avoided variation due to cells detaching during washing which occurred without fixation.

**Sucrose Density Gradients:** A linear gradient maker was loaded with 5.7 ml each of 5% and 20% sucrose in 0.5% Triton X-100 to construct an 11.4 ml, linear 5-20% sucrose gradient. Gradients were built in Beckman Quick-Seal centrifuge tubes (13x51 mm). 400  $\mu$ l of cell extract were combined with 2  $\mu$ l of extract of the electric organ from *Torpedo californica* (~1  $\mu$ M  $\alpha$ Bgt binding sites) for an internal size marker and loaded onto the top of each gradient. Gradients were centrifuged at 40,000 rpm for 16 hours at 4 $^{\circ}$  C in a Beckman XL-90 ultracentrifuge using a Beckman SW-41 rotor. After ultracentrifugation, the tubes were punctured and fractions were collected from the bottom into mAb coated Immulon flatbottom 4HBX wells using a fraction collector set to collect 10 drops per well (approx 130  $\mu$ l/well). 96 well Immulon 4HBX plates were coated with mAb 295 to bind AChRs containing  $\beta$ 2 or with mAb 338 to bind AChRs containing  $\beta$ 4. Fractions were bound to their respective antibody overnight at 4 $^{\circ}$  C with gentle agitation. After incubation, 30  $\mu$ l from each fraction were transferred onto an Immulon plate coated with mAb 210 to bind *Torpedo* AChRs, and 70  $\mu$ l of 2% Triton X-100 in Buffer A were

added. Solid phase radioimmuno assays were probed for 2 hours at room temperature with agitation with 2 nM [ $^{125}$ I]  $\alpha$ Bgt to detect *Torpedo californica* AChR binding on mAb 210 plates, or 2 nM [ $^3$ H] epibatidine to detect  $\alpha 6^*$  AChR binding on mAb 295 or mAb 338 plates. Then plates were washed 3 times with TPBS. Radioligand was dissociated by denaturing the samples with 50  $\mu$ l 0.1 N NaOH and transferred for counting into Costar 96 well white-walled plates containing 200  $\mu$ l of Optiphase SuperMix.

**Determining  $\beta 3$  Incorporation:**  $\alpha 6^*$  and  $\beta 3^*$  cell lines were extracted as above using Triton X-100 in Buffer A. Extracts were aliquoted into different sets for liquid phase immunoprecipitation, total [ $^3$ H] epibatidine binding, and mAb 210 agarose resin depletion. Sets aliquoted for mAb 210 agarose resin depletion were incubated with 20  $\mu$ l of agarose resin coated with mAb 210 in a total volume of 100  $\mu$ l along with 2 nM [ $^3$ H] epibatidine. These samples were incubated overnight at 4  $^{\circ}$ C with constant agitation. Supernatant fluid was collected from samples after 5,000x g centrifugation for 5 minutes and then AChRs were precipitated with mAb 338. All samples were denatured with 50  $\mu$ l 0.1 N NaOH for 5 minutes and then shaken with 1 ml Optiphase SuperMix for 2 hours.  $\beta 3$  incorporation was calculated by the difference between the total and depleted samples over the total binding.

**Binding of [ $^3$ H] epibatidine to cells.** Cells expressing  $\alpha 6^*$  AChRs were grown in media as above on 35mm dishes. One day prior to assay, nicotine was added into the growth media at a final concentration of 10  $\mu$ M for  $\beta 3$ -containing cells and 100  $\mu$ M for others. Binding to living cells attached to 35 mm plates was done in DMEM at 4 $^{\circ}$ C for only 15 min with 1 nM [ $^3$ H] epibatidine to minimize ongoing upregulation and penetration of quaternary amines inside the

cells. To determine the internal pool of epibatidine binding sites, 1 mM of the membrane impermeable quaternary amine methylcarbamylcholine was added together with 1 nM [ $^3\text{H}$ ] epibatidine to inhibit binding to cell surface AChRs. Nonspecific labeling (around 1% of total) was determined by incubation with 100  $\mu\text{M}$  nicotine and subtracted from total binding. After incubation, the cells were detached using 1ml ice cold PBS with 5 mM EDTA and washed three times with 1ml ice cold PBS by centrifugation (5 min at 500x g) in Eppendorf tubes. The washed pellets were dissociated with 100  $\mu\text{l}$  of 0.1M NaOH, and bound radioactivity was determined in the same tubes using the scintillation counter with 1 ml per tube of scintillation fluid.

## RESULTS

**Construction of Stably Transfected tsA201 HEK Cell Lines Expressing  $\alpha 6\beta 2$ ,  $\alpha 6\beta 2\beta 3$ ,  $\alpha 6\beta 4$ , and  $\alpha 6\beta 4\beta 3$  AChRs.** First  $\alpha 6\beta 2$  and  $\alpha 6\beta 4$  lines were established. Then these were transfected with  $\beta 3$  to produce lines expressing  $\alpha 6\beta 2\beta 3$  and  $\alpha 6\beta 4\beta 3$  AChRs.

Total amounts of AChRs expressed were measured by [ $^3\text{H}$ ] epibatidine binding to cell membrane fractions (Fig. 1A). Expression of  $\alpha 6\beta 2$  AChRs (45 fmol/mg protein) was very low compared to  $\alpha 3\beta 2$  (200 fmoles/mg, Wang et al., 1998) or  $\alpha 4\beta 2$  AChR HEK cell lines (900 fmol/mg, Kuryatov et al., 2005). Co-expression with  $\beta 3$  doubled the amount of AChR, much as co-expression with  $\alpha 5$  doubled the amount of  $\alpha 3\beta 2$  AChRs (Wang et al., 1998).  $\alpha 6\beta 4$  expression was twice that of  $\alpha 6\beta 2$ . Similarly,  $\alpha 3\beta 4$  expression in a HEK line exceeded  $\alpha 3\beta 2$  expression by four fold (Wang et al., 1998). The increased expression of  $\beta 4$ -containing compared to  $\beta 2$ -containing AChRs reflects less efficient assembly of  $\beta 2$  subunits due to a microdomain near ACh

binding site (Salette et al., 2004), and results in increased sensitivity of  $\beta 2$ -containing AChRs to the molecular chaperone effects of nicotine which selectivity promote increased assembly of  $\beta 2$ -containing AChRs (Wang et al., 1998; Kuryatov et al., 2005; Fig 1). Co-expression with  $\beta 3$  increased  $\alpha 6\beta 4$  expression a further 6 fold. By contrast, co-expression of  $\alpha 5$  with  $\alpha 3\beta 4$  decreased expression by 30% (Wang et al., 1998).

Incubation with 100  $\mu$ M nicotine overnight increased the amount of  $\alpha 6^*$  AChRs (Fig. 1A). This increased  $\alpha 6\beta 2$  AChRs 4 fold,  $\alpha 6\beta 2\beta 3$  AChRs 3.3 fold,  $\alpha 6\beta 4$  AChRs 3.2 fold, and  $\alpha 6\beta 4\beta 3$  AChRs 1.5 fold. At the same time, upregulation significantly increased surface expression in  $\beta 2$ -containing cells (Fig. 1B). After upregulation a greater fraction of the AChRs was found inside the cells (Fig. 1C). This was also observed after nicotine-induced upregulation of  $\alpha 4\beta 2$  AChRs (Kuryatov et al., 2005). Nicotine acts as a molecular chaperone to rapidly promote assembly of new  $\alpha 4\beta 2$  AChRs in the endoplasmic reticulum (Kuryatov et al., 2005; Salette et al., 2005; Corringer et al., 2006). Transport of  $\alpha 4\beta 2$  AChRs to the surface through the Golgi apparatus for modification of glycosylation may be a rate limiting step in surface expression. Similar processes may occur with  $\alpha 6^*$  AChRs.

The time course of upregulation by 100  $\mu$ M nicotine was similar for all of the cell lines (Fig. 2). Upregulation was half complete within 3 hours and close to maximal by 24 hours. This is similar to the kinetics of upregulation of  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  AChRs in tsA201HEK cell lines (Wang et al., 1998; Kuryatov et al., 2005).

**Raising mAbs to  $\alpha 6$  and  $\beta 3$  Subunits.** Mouse mAbs were raised to bacterially-expressed human  $\alpha 6$  and  $\beta 3$  subunits. Those mAbs which cross reacted between subunits by dot blots of bacterially-expressed subunits were eliminated, leaving 6 mAbs to  $\alpha 6$  and



one to  $\beta 3$  which were subunit-specific by this criterion (Fig. 3A). mAb 376 reacted only on blots with denatured  $\beta 3$ , not with native AChRs. The mAbs to  $\alpha 6$  also reacted on western blots with  $\alpha 6$  subunits from AChRs expressed in HEK cell lines and showed no crossreaction with  $\alpha 3$ ,  $\alpha 4$ ,  $\beta 2$ , or  $\beta 4$  subunits of AChRs expressed in these cell lines (Fig. 3B). mAb 349 was the best for use on western blots.

The relative amounts of  $\alpha 6$  subunits in the four cell lines were compared by western blotting (Fig. 3C). The  $\alpha 6\beta 2$  cell line expressed few native AChRs (Fig. 1A) and correspondingly contained few  $\alpha 6$  subunits (Fig. 3C). Thus, there were not large pools of unassembled  $\alpha 6$  subunits in the  $\alpha 6\beta 2$  line. The fact that transfection with  $\beta 3$  resulted in a greatly increased amount of  $\alpha 6$  as seen in the  $\alpha 6\beta 2\beta 3$  cell line suggests that unassembled  $\alpha 6$  subunits and  $\alpha 6\beta 2$  AChRs turn over rapidly and that the presence of  $\beta 3$  subunits permits the formation of  $\alpha 6\beta 2\beta 3$  AChRs which are much more stable and therefore much more  $\alpha 6$  accumulates.

The transmembrane orientation of the epitopes of the  $\alpha 6$  mAbs was determined by western blots (Fig. 3D). All six mAbs to  $\alpha 6$  on western blots recognized a chimera ( $\alpha 3/6$ ) with the extracellular domain of  $\alpha 3$  and the remainder of  $\alpha 6$ , but not a chimera ( $\alpha 6/3$ ) with the extracellular domain of  $\alpha 6$  and the remainder of  $\alpha 3$  (Fig. 3D). This indicates that the epitopes recognized by the mAbs are located C-terminal of the extracellular domain in the  $\alpha 6$  subunit.

The 6 mAbs to bacterially-expressed (denatured)  $\alpha 6$  also immunoprecipitate native human  $\alpha 6$  AChRs, with mAbs 338, 350, and 351 being the most potent (Table 1). Three of these mAbs also cross-reacted weakly with AChRs from rat brains. This was tested using high (25 $\mu$ g) amounts of mAbs in a 200  $\mu$ l reaction mix containing 0.2 nM

[<sup>3</sup>H] epibatidine labeled rat brain AChRs precipitable by mAb 295 to  $\beta$ 2 subunits. Of these AChRs, 4% were bound by mAb 350, and about 0.25% were bound by mAbs 338 and 351.

Immune adsorption demonstrated that  $\beta$ 3 subunits were incorporated into  $\alpha$ 6 AChRs (Fig. 4), as would be expected from the large increase of expression in the presence of  $\beta$ 3 (Fig. 1). The putative main immunogenic region (MIR) sequence 66-76 of human  $\alpha$ 1 subunits (KWNPPDDYGGVK) is closely related to sequences on human  $\alpha$ 3 (KWNPSDYGGAE),  $\alpha$ 5 (RWNPDDYGGIK), and  $\beta$ 3 (RWNPDDYGGIH). mAb 210 was made to muscle type AChRs, competes for binding to them with other mAbs to the MIR, and also binds to denatured  $\alpha$ 1 (Lindstrom, 2000). In addition it binds very well to native human  $\alpha$ 3 AChRs (Wang et al., 1998), but does not bind well to denatured  $\alpha$ 3. mAb 210 also binds to native but not denatured human  $\alpha$ 5 (Kuryatov et al., 1997). mAb 210 exhibited low affinity for direct immune precipitation of  $\alpha$ 6 $\beta$ 2 $\beta$ 3 or  $\alpha$ 6 $\beta$ 4 $\beta$ 3 AChRs, but mAb 210 coupled to agarose efficiently adsorbed  $\alpha$ 6 $\beta$ 2 $\beta$ 3 and  $\alpha$ 6 $\beta$ 4 $\beta$ 3 AChRs. It did not bind  $\alpha$ 6 $\beta$ 2 or  $\alpha$ 6 $\beta$ 4 AChRs. Figure 4 shows that 40% of  $\alpha$ 6 $\beta$ 2 $\beta$ 3 and 60% of  $\alpha$ 6 $\beta$ 4 $\beta$ 3 AChRs precipitated by mAb 338 could be adsorbed by mAb 210 coupled to agarose. This probably underestimates the actual percent of these AChRs which contain  $\beta$ 3 due to the low affinity of mAb 210 for  $\beta$ 3 in these  $\alpha$ 6 AChRs. Since mAb 210 binds efficiently only to native  $\alpha$ 3,  $\alpha$ 5, and  $\beta$ 3, but to both native and denatured  $\alpha$ 1, probably both the sequence and, especially, the conformation of this MIR epitope are important for binding of mAb210 to neuronal AChRs.

**Sedimentation of  $\alpha$ 6\*AChRs on Sucrose Gradients.** Previously we observed that, when expressed in *Xenopus* oocytes,  $\alpha$ 3 $\beta$ 2 AChRs sedimented on sucrose gradients

as a uniform 11 S component intermediate in size between monopentamers and dipentamers of *Torpedo* electric organ  $\alpha 1$  AChRs, whereas  $\alpha 6\beta 2$  AChRs assembled efficiently to form epibatidine binding sites within the oocytes but these sedimented as large amorphous aggregates (Kuryatov et al., 2000). When expressed in HEK cells, the  $\alpha 6\beta 2$  combination results in only small amounts of epibatidine binding sites, but most of these are on the cell surface (Fig. 1). Most of these  $\alpha 6\beta 2$  AChRs sediment as components larger than 11 S (Fig. 5), and substantial amounts pelleted in the centrifuge tube. Monopentamers were not clearly resolved from aggregates. Thus  $\alpha 6\beta 2$  AChRs do not efficiently form stable pentamers and may partially dissociate and aggregate. This disruption probably occurs during solubilization in Triton-X-100 because the efficient expression of  $\alpha 6\beta 2$  AChRs on the cell surface (Fig. 1C) suggests that in the membrane most were pentamers able to pass quality control analysis for exit from the endoplasmic reticulum and transport through the Golgi apparatus to the cell surface. Incorporation of the  $\beta 3$  subunit to form  $\alpha 6\beta 2\beta 3$  AChRs not only results in assembly of many more AChRs (Fig. 1), but also most of these sediment as a component which is nearly the size of 9.5 S *Torpedo* AChR monopentamers (Fig. 5). Thus, the presence of  $\beta 3$  accessory subunits permits the efficient assembly of stable pentameric AChRs. Both  $\alpha 6\beta 4$  and  $\alpha 6\beta 4\beta 3$  AChRs expressed in the cell lines sediment as a mixture of a component corresponding to monopentamers and larger aggregates (Fig. 5). The larger proportion of aggregates may bias the monopentamer peak to appear to sediment more rapidly than in the case of  $\alpha 6\beta 2\beta 3$  AChRs. The formation of aggregates on sucrose gradients which is prominent with  $\alpha 6^*$  AChRs is not observed with  $\alpha 1^*$ ,  $\alpha 3\beta 2$ , or  $\alpha 4\beta 2$  AChRs (Kuryatov et al., 2000; 2005).

**Agonist binding to  $\alpha 6$  AChRs.** Figure 6 shows the concentration dependence of [ $^3$ H] epibatidine binding to all four  $\alpha 6^*$  AChR subtypes. The  $K_D$  for binding of epibatidine to  $\alpha 6\beta 2$  AChRs (0.154 nM) was basically the same as the  $K_D$  for binding to  $\alpha 6\beta 2\beta 3$  AChRs (0.198 nM),  $\alpha 6\beta 4$  AChRs (0.162 nM), or  $\alpha 6\beta 4\beta 3$  AChRs (0.130 nM). This compares to the  $K_D$  for binding of epibatidine to human  $\alpha 4\beta 2$  AChRs similarly expressed and measured of 0.017 nM (Kuryatov et al., 2005). Thus, the presence of  $\beta 3$  does not alter equilibrium binding affinity of  $\alpha 6\beta 2^*$  or  $\alpha 6\beta 4^*$  AChRs for epibatidine. All four  $\alpha 6^*$  AChR subtypes exhibit much lower affinity for epibatidine than do  $\alpha 4\beta 2$  AChRs.

Agonists were tested for their ability to inhibit the binding of [ $^3$ H] epibatidine to fixed cells (Table 2). In general, the  $\beta 2$ -containing  $\alpha 6^*$  AChRs exhibited higher affinities for agonists. Cytisine discriminated by more than 7 fold in affinity between  $\alpha 6\beta 2^*$  and  $\alpha 6\beta 4^*$  AChRs. The affinity for nicotine is not significantly altered by the presence or absence of  $\beta 3$  subunits.

Both equilibrium-binding studies of epibatidine (Fig. 6) and competitive binding studies with nicotine and other ligands (Table 2) indicate that the presence of  $\beta 3$  does not alter the ACh binding site or its affinity for nicotine. This is not surprising because  $\beta 3$  assembles in the accessory position and is not part of an ACh binding site.

**$\beta 3$  Greatly Increased Sensitivity of  $\alpha 6^*$  AChRs to Nicotine-Induced Upregulation.** Table 3 shows that the presence of  $\beta 3$  increased the sensitivity to nicotine-induced upregulation of  $\alpha 6\beta 2\beta 3$  by 11 fold compared to  $\alpha 6\beta 2$  AChRs and of  $\alpha 6\beta 4\beta 3$  AChRs by 6.6 fold compared to  $\alpha 6\beta 4$  AChRs. The binding data of Fig. 6 and the Table 2 show that the presence of  $\beta 3$  does not alter the ACh binding sites and greatly increase

affinity for nicotine. So how might  $\beta 3$  have such a large effect on nicotine-induced upregulation? Nicotine probably acts on  $\alpha 6^*$  AChRs as a molecular chaperone, as it does on  $\alpha 4\beta 2$  AChRs (Kuryatov et al., 2005; Sallette et al., 2005). Binding of nicotine to  $\alpha 6\beta 2\alpha 6\beta 2$  or  $\alpha 6\beta 4\alpha 6\beta 4$  assembly intermediates could produce activated or desensitized conformations which would assemble more efficiently with  $\beta 3$  than  $\beta 2$  or  $\beta 4$  subunits in the accessory position in a final assembly step to form mature pentamers. The greater AChR expression observed with  $\beta 3$ -containing AChRs in the absence of nicotine (Fig. 1) shows that  $\beta 3$  promotes assembly of mature AChRs. Alternatively, nicotine could act as a molecular chaperone on  $\alpha 6\beta 2\beta 3$  or  $\alpha 6\beta 4\beta 3$  assembly intermediates and the presence of  $\beta 3$  could promote conformation changes to the active or desensitized conformations which assemble more efficiently. The accessory subunit  $\alpha 5$  influences the sensitivity to activation and desensitization of  $\alpha 3^*$  AChRs (Gerzanich et al., 1998).

The  $EC_{50}$  for upregulation by nicotine of  $\alpha 6\beta 2$  AChRs (9.8  $\mu M$ ) indicates that at the 0.1-0.2  $\mu M$  concentrations of nicotine sustained in the sera of cigarette smokers (Benowitz, 1996) upregulation of this subtype would be negligible. By contrast, the  $EC_{50}$  for  $\alpha 6\beta 2\beta 3$  (0.89  $\mu M$ ) reveals that the presence of  $\beta 3$  confers an order of magnitude more sensitivity to nicotine-induced upregulation, sufficient to suggest that some upregulation might occur in a smoker. Still, the sensitivity is much less than that of human  $\alpha 4\beta 2$  AChRs expressed in tsA201 HEK cells ( $EC_{50} = 0.039 \mu M$ ) (Kuryatov et al., 2005), an AChR subtype which has much higher affinity for nicotine ( $K_D = 0.0028 \mu M$ ).  $\beta 3$  also increases the sensitivity to nicotine-induced upregulation of  $\alpha 6\beta 4\beta 3$  AChRs from  $EC_{50} = 3.55 \mu M$  for  $\alpha 6\beta 4$  AChRs to  $EC_{50} = 0.54 \mu M$  for  $\alpha 6\beta 4\beta 3$  AChRs.

Unlike human  $\alpha 3$  AChRs expressed in HEK cell lines, where only  $\beta 2$ -containing but not  $\beta 4$  containing AChRs are subject to nicotine-induced upregulation (Wang et al., 1998),  $\alpha 6^*$  AChRs containing either  $\beta 2$  or  $\beta 4$  subunits are sensitive to nicotine-induced upregulation. The extent of upregulation is greater for  $\alpha 6\beta 2\beta 3$  AChRs than for  $\alpha 6\beta 4\beta 3$  AChRs, presumably reflecting the lower base line assembly efficiency of  $\beta 2$  than  $\beta 4$  subunits (Wang et al., 1998; Sallette et al., 2004).

### **Culture at 29 °C in Combination with Nicotine Dramatically Upregulates**

**Expression of  $\alpha 6\beta 2$  AChRs.** Cooper et al. (1999) initially observed that culture at 30 °C increased expression of  $\alpha 4\beta 2$  AChRs, and proposed that this resulted from increased assembly and/or slower turnover. The low amount of  $\alpha 6$  subunits in the  $\alpha 6\beta 2$  cell line by contrast with the large amount after transfection of this line with  $\beta 3$  (Fig. 3C) suggests that unassembled  $\alpha 6$  and  $\alpha 6\beta 2$  AChRs are unstable and that transfection with  $\beta 3$  promotes assembly and/or stabilizes the resulting AChRs. The large amount of  $\alpha 6$  in the other lines shows that, if the AChRs are stabilized by the right subunit combination and ambient condition, the  $\alpha 6$  expression vector can result in substantial amounts of AChRs. Culture at 29 °C increased the amount of  $\alpha 6\beta 2$  AChRs to that obtained in the presence of  $\beta 3$ , and greatly increased the extent and sensitivity to nicotine-induced upregulation, somewhat exceeding the effect of  $\beta 3$  on the amount of AChR (Fig. 7).  $\alpha 6\beta 4$  AChRs were expressed at a higher level than  $\alpha 6\beta 2$  at 37 °C, so the effects of culture at 29 °C were substantial but less. In the presence of  $\beta 3$ , there was little effect on nicotine-induced upregulation of  $\alpha 6\beta 2\beta 3$  AChRs and virtually none on  $\alpha 6\beta 4\beta 3$  AChRs. These results are consistent with the idea that low temperature greatly increases the stability of  $\alpha 6\beta 2$  assembly intermediates and AChRs, and increases to a lesser extent the stability of

the intrinsically more stable  $\alpha 6\beta 4$  assembly intermediates and AChRs, while also promoting their assembly synergistically with the pharmacological chaperone effects of nicotine.

The differences in  $\alpha 6\beta 2$  expression between HEK cells shown here and *Xenopus* oocytes (Kuryatov et al., 2000) demonstrate the importance of cell-specific factors on AChR assembly, and suggest the possibility that in some cell types (e.g. the aminergic neurons in which  $\alpha 6^*$  AChRs are exclusively found *in vivo*) chaperone proteins might be able to produce effects equivalent to culture at 29 °C.

## DISCUSSION

Four subtypes of human  $\alpha 6^*$  AChRs ( $\alpha 6\beta 2$ ,  $\alpha 6\beta 2\beta 3$ ,  $\alpha 6\beta 4$ , and  $\alpha 6\beta 4\beta 3$ ) were stably expressed in human tsA201 HEK cell lines, and six mAbs to human  $\alpha 6$  and one mAb to human  $\beta 3$  subunits were made to aid in the characterization of  $\alpha 6^*$  AChRs. These  $\alpha 6$  mAbs were found to be directed at sequences C-terminal of the extracellular domain. These lines will permit more detailed studies of these AChR subtypes than is possible with brain neurons containing small amounts of complex mixtures of AChR subtypes. Even more complex  $\alpha 6^*$  AChR subtypes such as  $\alpha 6\alpha 4\beta 2\beta 3$  which have been identified in brain and retina (Zoli et al., 2002; Champtiaux et al., 2003; Gotti et al., 2005a,b) will also need to be studied in transfected cell lines.  $\alpha 6^*$  AChRs are potential drug targets, for example in Parkinson's disease (Quik and McIntosh, 2006).

Host cell type influences the expression of AChRs, presumably as a reflection of differences the complement of chaperone proteins and enzymes for post-translational

modification. For example, the chaperone protein Ric-3 is expressed in the human neuroblastoma cell line SH-SY5Y, which endogenously expresses  $\alpha 7$  AChRs, but is not expressed in tsA201 HEK cells, which efficiently express transfected  $\alpha 7$  AChRs only after co-transfection with Ric-3 (Williams et al., 2005; Landsdell et al., 2005).  $\alpha 6^*$  AChRs have only been reported in aminergic neurons which may contain particular chaperones for the assembly and transport of  $\alpha 6^*$  AChRs.

Expression of  $\alpha 6\beta 2$  in *Xenopus* oocytes results in the formation of large amounts of epibatidine binding sites but no mature pentameric AChRs on the cell surface (Kuryatov et al., 2000). Instead,  $\alpha 6$  and  $\beta 2$  subunits form amorphous intracellular aggregates. In HEK cells only small amounts of  $\alpha 6\beta 2$  AChRs are made, but a large proportion are on the cell surface.  $\alpha 6$  is closely related in sequence to  $\alpha 3$  (Lindstrom, 2000; LeNovere et al., 2002).  $\alpha 3\beta 2$  forms functional AChRs in oocytes (Gerzanich et al., 1998). In tsA201 HEK cell lines,  $\alpha 3\beta 2$  expresses less well than does  $\alpha 3\beta 4$  (Wang et al., 1998), resembling the relationship between  $\alpha 6\beta 2$  and  $\alpha 6\beta 4$ . In HEK cell lines,  $\alpha 3\beta 2$  is greatly upregulated by nicotine but human  $\alpha 3\beta 4$  is not (Wang et al., 1998).  $\alpha 6\beta 4$  is upregulated by nicotine. Thus, there are both cell type and subunit-specific factors which influence expression.

Culture at 29 °C dramatically increases the expression of  $\alpha 6\beta 2$  and its sensitivity to upregulation by nicotine, while having a smaller effect on  $\alpha 6\beta 4$  (that is intrinsically expressed at a higher level). The low temperature apparently greatly stabilizes  $\alpha 6\beta 2$  AChRs or their assembly intermediates, permitting the accumulation of large amounts under the influence of nicotine. Nicotine probably acts as a pharmacological chaperone



(Kuryatov et al., 2005; Corringer et al., 2006). In HEK cells, nicotine dramatically increases the amount of  $\alpha 3\beta 2$  AChRs (22 fold) without causing a dramatic increase (perhaps 2 fold) in the amount of  $\alpha 3$  subunits on western blots (Wang et al., 1998), indicating that, as in an  $\alpha 4\beta 2$  line (Kuryatov et al., 2005), there are large pools of unassembled  $\alpha 3$  subunits present. By contrast, western blots of  $\alpha 6\beta 2$  reveal that only small amounts of  $\alpha 6$  are present, even though transfection of this line with  $\beta 3$  results in much larger amounts of  $\alpha 6$ , and parallel  $\alpha 6\beta 4$  and  $\alpha 6\beta 4\beta 3$  lines contain much more  $\alpha 6$ . Thus unassembled  $\alpha 6$  as well as  $\alpha 6\beta 2$  AChRs must be relatively unstable. Using chimeras between  $\alpha 6$  and  $\alpha 3$  or  $\alpha 4$  subunits, the part of the  $\alpha 6$  subunit which limits expression of mature AChRs with  $\beta 2$  in *Xenopus* oocytes was mapped to sequences C-terminal of the extracellular domain of  $\alpha 6$  (Kuryatov et al., 2000). In  $\alpha 6^*$  AChR-expressing neurons there may be chaperones which have effects similar to culture at 29 °C in promoting the assembly and stability of  $\alpha 6\beta 2$  AChRs.

The presence of  $\beta 3$  in the accessory position greatly increased expression of  $\alpha 6\beta 2$  and  $\alpha 6\beta 4$ , increased sensitivity to upregulation by nicotine, and negated any additional effect of culture at 29 °C. In these respects, the effects of  $\beta 3$  were more dramatic than the effects of  $\alpha 5$  on  $\alpha 3$  AChRs (Gerzanich et al., 1998; Wang et al., 1998). The effects of temperature, nicotine, and subunit composition suggest that in these cell lines regulation of AChR expression occurs primarily at the post translational level. Studies of the amount of AChR  $\alpha 4$  and  $\beta 2$  subunit protein in the brains of AChR subunit knockout mice similarly demonstrate that in brain, the regulation of AChR expression also occurs primarily at the post translational level (Whiteaker et al., submitted). In both *Xenopus*

oocytes (Peng et al., 1994), HEK cells (Kuryatov et al., 2005) and rodent brain (Flores et al., 1992) nicotine-induced upregulation of  $\alpha 4\beta 2$  AChRs occurs by post-translational mechanisms.

The complete aggregation of  $\alpha 6\beta 2$  AChRs expressed in *Xenopus* oocytes (Kuryatov et al., 2000) and extensive aggregation of  $\alpha 6^*$  AChR subtypes expressed in HEK cells suggests that this reflects a particular property of  $\alpha 6$  subunits. The detergent Triton X-100 used for solubilization may partially dissociate some  $\alpha 6$  AChRs subtypes and subsequent re-aggregation might account for the sedimentation properties observed. The presence of  $\beta 3$  greatly increased the proportion of monopentamers in  $\alpha 6\beta 2\beta 3$  AChRs compared to  $\alpha 6\beta 2$  AChRs, perhaps by stabilizing them against dissociation. It will be interesting to determine if substitution of one of the two  $\alpha 6$  subunits in an AChR pentamer by an  $\alpha 4$  or  $\alpha 3$  subunit to produce the most abundant and complex subtypes of brain  $\alpha 6^*$  AChRs might reduce or eliminate aggregation of the resulting AChRs, reflecting a stabilizing influence on pentamers.

The presence of  $\beta 3$  subunits increased sensitivity to upregulation by nicotine of  $\alpha 6^*$  AChRs to the range where the 0.1-0.2  $\mu\text{M}$  concentration of nicotine sustained in the sera of cigarette smokers (Benowitz, 1996) would be expected to cause some upregulation of these  $\alpha 6^*$  AChR subtypes in brain. However, these  $\alpha 6^*$  AChRs are much less sensitive to upregulation than are  $\alpha 4\beta 2$  AChRs (Kuryatov et al., 2005).

Parker et al. (2004) reported selective upregulation of  $\alpha 6\beta 2$  AChRs compared to  $\alpha 4\beta 2$  AChRs in rat brain as a result of long term self-administration of nicotine. On the contrary, reduction in the amount of  $\alpha 6^*$  AChRs after nicotine treatment in various other

ways has been reported in rats, mice, and monkeys (Lai et al., 2005; McCallum et al., 2006; Mugnaini et al., 2006). How can these apparently contradictory results on the ability of nicotine to upregulate  $\alpha 6^*$  AChRs be explained? Dopaminergic neurons in the ventral tegmental area of rodents express a mixture of  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$ , and  $\beta 3$  subunits and preferentially transport  $\alpha 4\alpha 6\beta 2\beta 3$  to their nerve endings in the striatum while expressing  $\alpha 4\beta 2$  AChRs on the cell bodies (Zoli et al., 2002; Champtiaux et al., 2003; Gotti et al., 2005b; Quik et al., 2005). In primates,  $\alpha 3$  subunits are also involved and there is a lower proportion of  $\alpha 4\beta 2$  in the endings. In monkey striatum 70% of nicotine-evoked dopamine release is mediated by  $\alpha 6\beta 2^*$  and/or  $\alpha 3\beta 2^*$  AChRs, whereas in rodents  $\alpha 6\beta 2^*$  AChRs mediate only 30% (Quik and McIntosh, 2006). This complex mixture of AChRs subunits competes for assembly in the endoplasmic reticulum of these neurons. Nicotine acts as a molecular chaperone to promote assembly of AChRs by binding to assembly intermediates (Kuryatov et al., 2005; Sallette et al., 2005; Corringier et al., 2006). The mean plasma nicotine concentration in monkeys given nicotine in their drinking water was 51 nM (McCallum et al., 2006). This would efficiently promote upregulation of human  $\alpha 4\beta 2$  AChRs expressed in HEK cells ( $EC_{50}=35$  nM, Kuryatov et al. (2005)), but would have little effect on  $\alpha 6\beta 2\beta 3$  AChRs ( $EC_{50}=890$  nM). If the amount of  $\beta 2$  subunits were limiting, at low nicotine concentrations, increased assembly of  $\alpha 4\beta 2$  AChRs could decrease assembly of  $\alpha 6\beta 2\beta 3$  AChRs by depleting the pool of  $\beta 2$  subunits. Nicotine induced slowing of mature AChR destruction (Kuryatov et al., 2005) could also upregulate AChRs in nerve endings as well as on the cell body. Upregulation by this mechanism might also selectively affect high affinity  $\alpha 4\beta 2^*$  AChRs. Most nicotine-induced brain AChR upregulation is of highly sensitive  $\alpha 4\beta 2$  AChRs (Flores et al.,

1992). Autonomic ganglia express a mixture of  $\alpha 3$ ,  $\beta 2$ ,  $\beta 4$ , and  $\alpha 5$  subunits, but preferentially assemble  $\alpha 3\beta 4$  AChRs (Davila-Garcia et al., 2003; Nguyen et al., 2003). The nicotine concentrations obtained in vivo result in negligible upregulation of the less sensitive  $\alpha 3^*$  AChRs.

The large increase in amount of  $\alpha 6^*$  AChRs as well as their sensitivity to nicotine-induced upregulation which results from the  $\beta 3$  subunit are consistent with the hypothesis that assembly of the  $\beta 3$  subunit in the accessory position is the final step in assembly of mature AChRs.  $\beta 3$  subunit appears to preferentially assemble in the accessory position over  $\beta 2$  or  $\beta 4$  to result in larger amounts of AChRs.  $\beta 3$ -containing  $\alpha 6$  AChRs did not show increased affinity for nicotine. The increased sensitivity to upregulation by nicotine in the presence of  $\beta 3$  could result from assembly intermediates such as  $\alpha 6\beta 2$  or  $\alpha 6\beta 2\alpha 6\beta 2$  assuming a nicotine-induced desensitized conformation which more efficiently assembles with  $\beta 3$ . Alternatively, assembly intermediates incorporating  $\beta 3$  such as  $\beta 3\alpha 6\beta 2$  could more efficiently assume a nicotine-induced conformation which assembles more efficiently.

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## FOOTNOTES

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## LEGENDS FOR FIGURES

**Fig. 1.** Extent of  $\alpha 6^*$  AChR expression in transfected cell lines. A. Total AChRs were determined by 2nM [ $^3\text{H}$ ] epibatidine binding to membrane fragments of the four cell lines expressing different  $\alpha 6^*$  AChR subtypes. Binding was assayed under control conditions and after culture overnight in medium containing 100  $\mu\text{M}$  nicotine to maximally upregulate the amount of AChR. B. Cell surface AChRs were determined by measuring the fraction of 2 nM [ $^3\text{H}$ ] epibatidine binding to intact cells which was inhibited by the presence of 10 mM methylcarbamylcholine. The tertiary amine epibatidine rapidly crosses the cell membrane to label AChRs both on the surface and interior of cells, but the quaternary amine methylcarbamylcholine crosses the membrane so slowly that during the 1 hour assay it binds almost exclusively to AChRs on the cell surface (Kuryatov et al., 2005). C. Data were plotted to indicate the fraction of AChRs on the cell surface under control conditions and after upregulation overnight by 100  $\mu\text{M}$  nicotine. Bars show measured values  $\pm$  S.E.M. for quadruplicate samples.

**Fig. 2.** Time course of nicotine-induced upregulation of  $\alpha 6^*$  AChRs. Cells were grown on 24 well tissue culture plates. All cells were grown for the same total time and fixed at the same time before assay. Nicotine (100  $\mu\text{M}$ ) was added at various times prior to fixation. The specific binding of 2 nM [ $^3\text{H}$ ] epibatidine was determined. Specific binding for parallel culture not treated with nicotine was subtracted. Then the amount of nicotine-induced binding for each line was expressed as a percent of the maximum obtained for each line. Figure 1A shows the different base lines and maximum extents of upregulation

for each line. Expressing the data for all lines as a percent of the maximum upregulation allows the kinetics of upregulation in all lines to be compared. Although the base line and extent of upregulation differed with each line, the time courses of upregulation for all were the same. The values shown are mean  $\pm$  S.E.M. for triplicate samples.

**Fig. 3.** Reaction of mAbs to  $\alpha 6$  and  $\beta 3$  with denatured subunits on blots. A. The subunit specificity of the mAbs was demonstrated by dot blot assays using bacterially-expressed human AChR subunit constructs. Reaction is compared to that of antiserum to each of the subunit constructs to demonstrate equal loading of the blots of all the subunits and thus the high specificity of the mAbs. B. The specificity of the mAbs was further evaluated using western blots of equal amounts of AChRs from HEK cell lines transfected with human  $\alpha 6\beta 4\beta 3$ ,  $\alpha 3\beta 4\alpha 5$  or  $\alpha 4\beta 2$  AChRs. The mAbs reacted strongly only with  $\alpha 6$  subunits and not at all with the other subunits present. C. The extent of expression of  $\alpha 6$  subunits in the four  $\alpha 6^*$  AChR cell lines was compared using an equal amount of protein from each line and mAb 349. mAbs were used at a 1/1000 dilution and [ $^{125}$ I] goat anti-mouse IgG purified antibodies were used at 2 nM. D. Reaction of mAb 353 with chimeras (Kuryatov et al., 2000) consisting of the extracellular domains of  $\alpha 3$  or  $\alpha 6$  in combination with the remainder of the other of these subunits on western blots. The transmembrane orientation of the epitopes of the  $\alpha 6$  mAbs was determined using western blots of equal amounts of chimeric AChRs from *Xenopus* oocytes injected with 25 ng per subunit of cRNA for the subunit combinations of  $\alpha 3/6\beta 2$  or  $\alpha 6/3\beta 2$ . All six mAbs to  $\alpha 6$  reacted strongly with chimeric  $\alpha 3/6$  subunits and not at all with  $\alpha 6/3$  subunits. Only the western blot of mAb 353 is shown.

**Fig. 4.** Incorporation of  $\beta 3$  into  $\alpha 6^*$  AChRs can be detected by immune depletion using mAb 210 to the MIR. mAb 338 to  $\alpha 6$  subunits was used to precipitate AChRs labeled with [ $^3\text{H}$ ] epibatidine before and after adsorption with mAb 210 coupled with agarose. The labeled AChRs coupled to mAb 210-agarose were eluted and counted as well. mAb 210 was made to muscle AChR and binds to the MIR on  $\alpha 1$  subunits which includes a sequence similar to that on  $\beta 3$  subunits. mAb 210 agarose does not bind to  $\alpha 6\beta 2$  or  $\alpha 6\beta 4$  AChRs (data not shown). The values shown are mean  $\pm$  S.E.M. for triplicate samples.

**Fig. 5.** Sucrose gradient sedimentation of  $\alpha 6^*$  AChRs. Sedimentation on SW41 5-20% sucrose gradients used *Torpedo* AChRs as internal standards. *Torpedo* AChRs were immunisolated from gradient fractions on wells coated with mAb 210, then labeled with [ $^{125}\text{I}$ ]  $\alpha$  bungarotoxin. The positions of the peaks corresponding to the 9.5 S monomers and 13 S dimers of *Torpedo* AChRs are indicated by arrows.  $\alpha 6\beta 2$  and  $\alpha 6\beta 2\beta 3$  AChRs were isolated on microwells coated with mAb 295 to  $\beta 2$  subunits.  $\alpha 6\beta 4$  and  $\alpha 6\beta 4\beta 3$  AChRs were isolated on microwells coated with mAb 338 to  $\alpha 6$  subunits. Isolated  $\alpha 6^*$  AChRs were labeled with [ $^3\text{H}$ ] epibatidine. mAb 338 to  $\alpha 6$  and mAb 337 to  $\beta 4$  subunits (Nelson et al., 2001) were equally effective when coated on microwells at adsorbing  $\alpha 6\beta 4$  and  $\alpha 6\beta 4\beta 3$  AChRs. However mAb 338 was much less effective at adsorbing  $\alpha 6\beta 2$  and  $\alpha 6\beta 2\beta 3$  AChRs. Thus mAb 295 coated wells were used. The epitope for mAb 338 is probably near the subunit interface and influenced by the  $\beta$  subunit present. The sucrose gradients shown were representative of several similar gradients analyzed.



**Fig. 6.** Concentration dependence of binding of [<sup>3</sup>H] epibatidine by cell lines.  $\alpha 6\beta 2^*$  AChR cells were fixed after upregulation overnight with nicotine (100  $\mu$ M in case of  $\alpha 6\beta 2$  or 10  $\mu$ M in case of  $\alpha 6\beta 2\beta 3$ ) to provide convenient amounts of AChRs for assay.  $\alpha 6\beta 4$  and  $\alpha 6\beta 4\beta 3$  cells did not require upregulation. Binding was assayed using the indicated concentrations of [<sup>3</sup>H] epibatidine. Background binding, assayed in the presence of 1mM nicotine, was subtracted. The values shown are mean  $\pm$  S.E.M. for quadruplicate assays.

**Fig. 7.** Culture at 29 °C greatly increased expression and sensitivity to nicotine-induced upregulation of  $\alpha 6\beta 2$  and  $\alpha 6\beta 4$  AChRs. The indicated cultures were shifted to 29 °C for the 12-15 hour period during which nicotine was applied. Then cells were fixed prior to measuring binding of [<sup>3</sup>H] epibatidine applied at 2 nM. The values shown are mean  $\pm$  S.E.M. for quadruplicate assays.

## TABLES

**Table 1. Immune Precipitation of Native [<sup>3</sup>H] Epibatidine Labeled Human AChRs by mAbs to  $\alpha 6$  Subunits.**

The cell lines expressing  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  AChRs were described previously (Wang et al., 1998 and Kuryatov et al., 2005). A 0.5 nM concentration of AChRs and 1.5 nM of [<sup>3</sup>H]-epibatidine were used in these assays using triplicate samples.

AChR	Titer of mAb ( $\mu\text{mol } ^3\text{H epibatidine binding sites/l mAb}$ )					
	mAb 338	mAb 339	mAb 349	mAb 350	mAb 351	mAb 353
$\alpha 3\beta 2$	0	0	0	0	0	0
$\alpha 4\beta 2$	0	0	0	0	0	0
$\alpha 6\beta 2$	4.1	0.09	0.3	5.7	6	0

**Table 2. Relative Affinities of  $\alpha 6^*$  AChR Subtypes for Agonists**

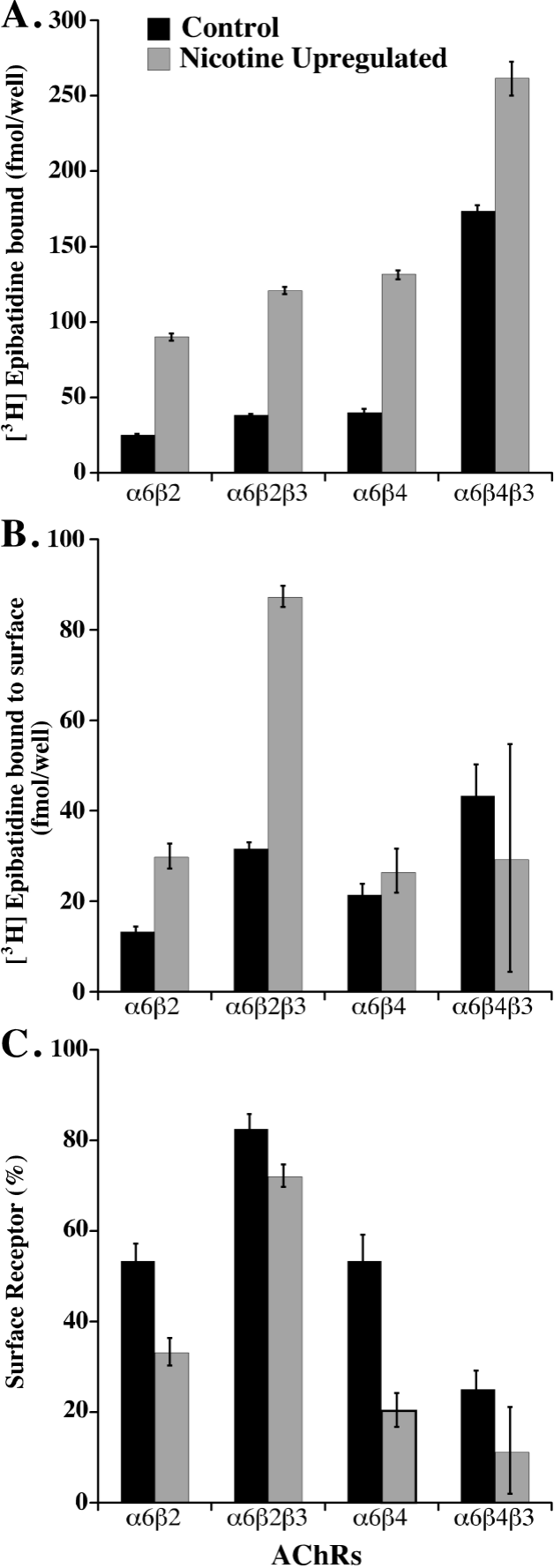
Serial dilutions of agonists were applied with [ $^3\text{H}$ ] epibatidine at 2 nM to cells fixed with formalin on Costar 96 well white with clear bottom plates. The concentration-response curves were fitted using a nonlinear least-squares error curve fit method (KaleidaGraph, Abelbeck software) to Hill equation:  $I(x) = I_{\max}x^n/(x^n+IC_{50}^n)$ . Values are shown  $\pm$ S.E.M.

<b>AChR</b>	<b>IC<sub>50</sub> (<math>\mu\text{M}</math>)</b>			
	<b>ACh</b>	<b>Nicotine</b>	<b>DMPP</b>	<b>Cytisine</b>
$\alpha 6\beta 2$	2.82 $\pm$ 1.04	0.72 $\pm$ 0.16	0.51 $\pm$ 0.01	0.25 $\pm$ 0.10
$\alpha 6\beta 2\beta 3$	1.52 $\pm$ 0.01	0.40 $\pm$ 0.05	0.93 $\pm$ 0.06	0.25 $\pm$ 0.01
$\alpha 6\beta 4$	6.30 $\pm$ 0.64	1.79 $\pm$ 0.17	1.02 $\pm$ 0.22	1.92 $\pm$ 0.28
$\alpha 6\beta 4\beta 3$	6.88 $\pm$ 0.24	1.39 $\pm$ 0.03	2.08 $\pm$ 0.12	4.48 $\pm$ 0.27

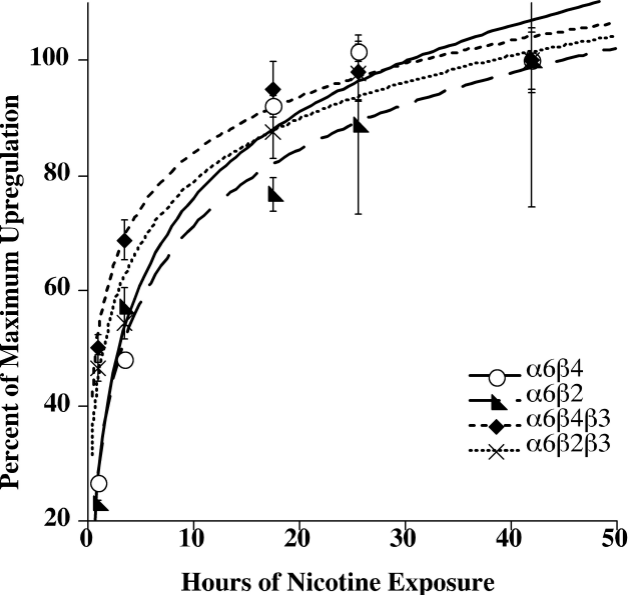
### Table 3. Sensitivity to Nicotine-Induced Upregulation

Serial dilutions of nicotine were added to cells close to confluency and the cells were incubated overnight on Costar 96 well white with clear bottom plates. The cells were fixed with formalin and incubated with 2 nM [<sup>3</sup>H] epibatidine. The concentration-response curves were fitted using a nonlinear least-squares error curve fit method (KaleidaGraph, Abelbeck software) to Hill equation:  $I(x) = I_{\max}x^n/(x^n+EC_{50}^n)$ . Values are shown  $\pm$ S.E.M.

<b>AChR</b>	<b>EC<sub>50</sub></b>	<b>Bmax</b>
	( $\mu$ M)	(fmol/well)
$\alpha$ 6 $\beta$ 2	9.84 $\pm$ 0.03	1.13 $\pm$ 0.08
$\alpha$ 6 $\beta$ 2 $\beta$ 3	0.89 $\pm$ 0.29	4.56 $\pm$ 0.75
$\alpha$ 6 $\beta$ 4	3.55 $\pm$ 0.62	21.3 $\pm$ 1.7
$\alpha$ 6 $\beta$ 4 $\beta$ 3	0.54 $\pm$ 0.13	42.0 $\pm$ 4.1

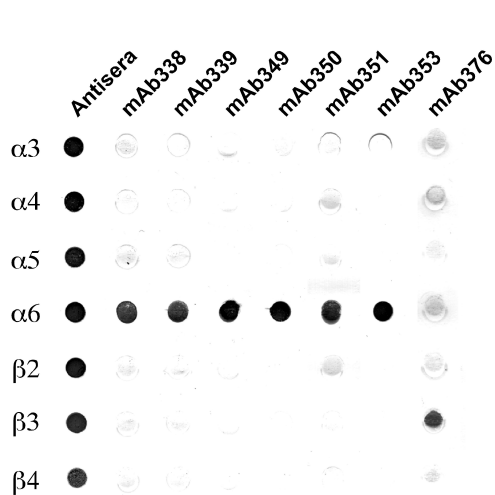


**Figure 1**

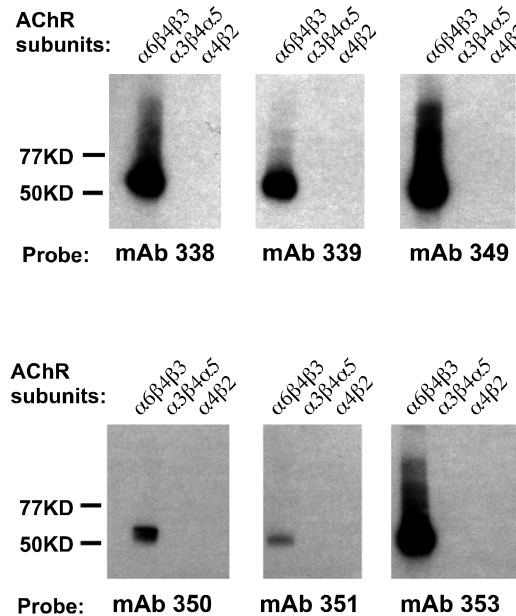


**Figure 2**

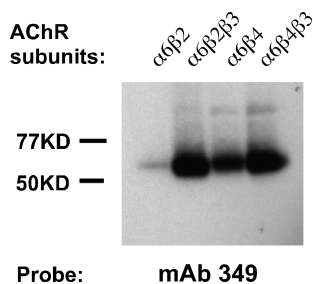
A



B



C.



D.

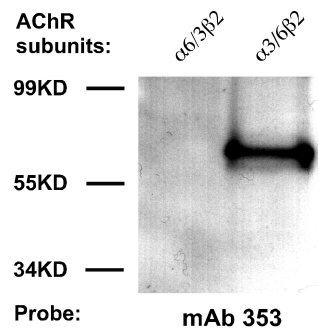
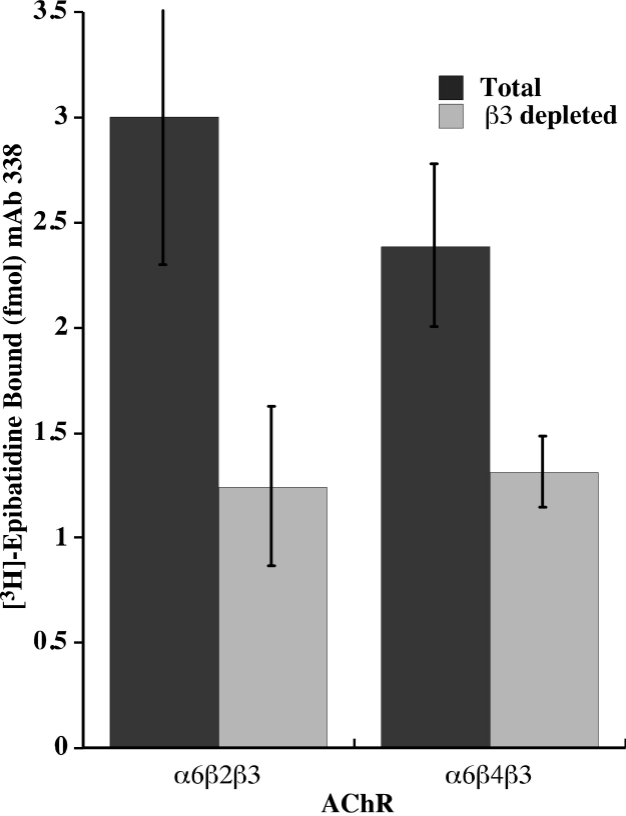


Figure 3



**Figure 4**



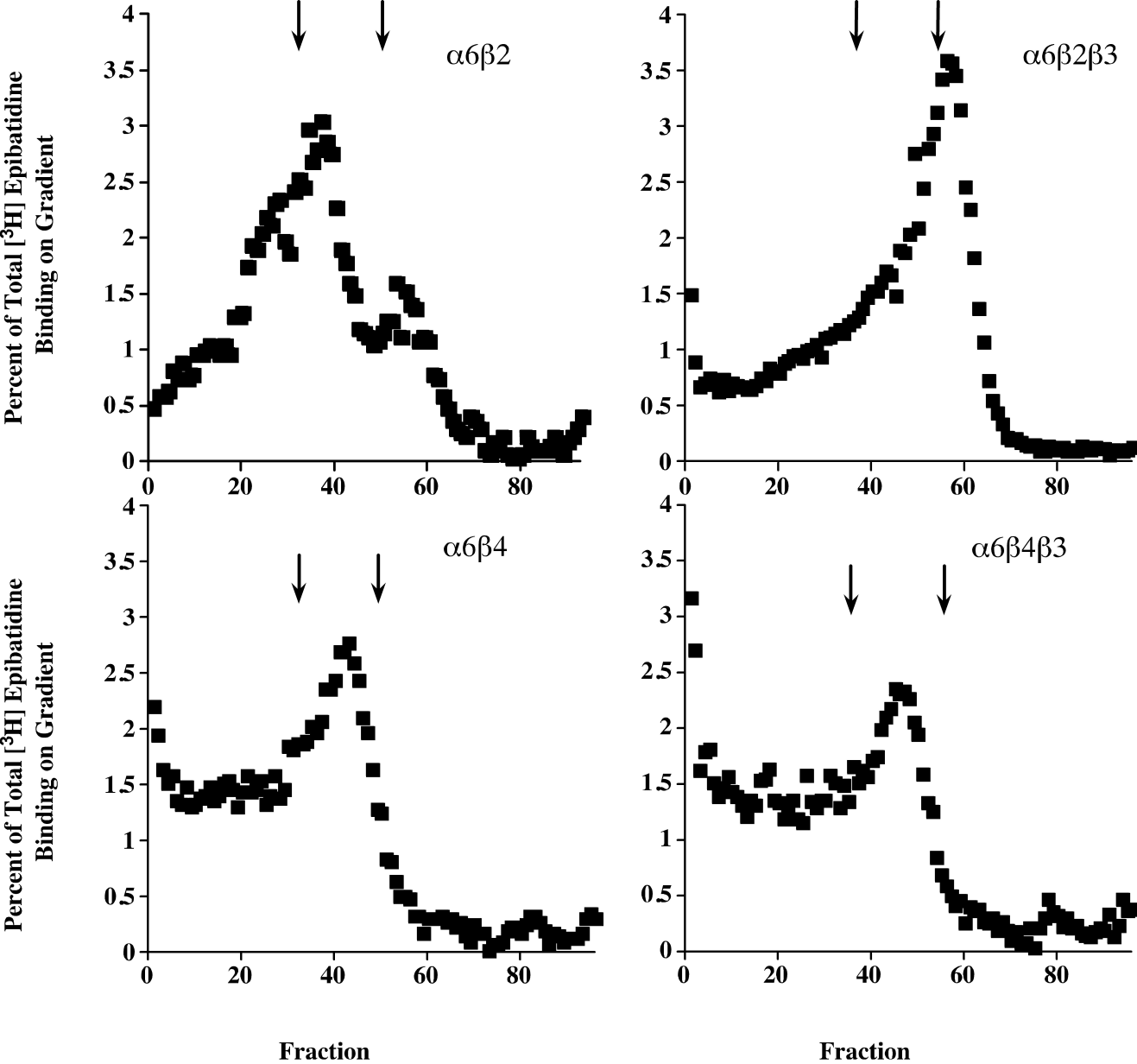
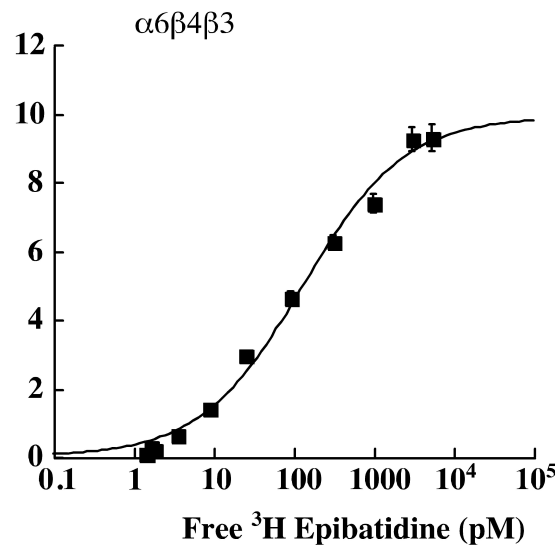
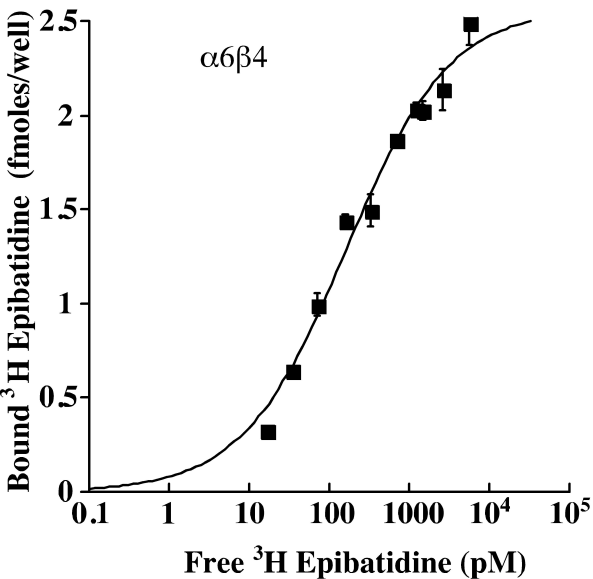
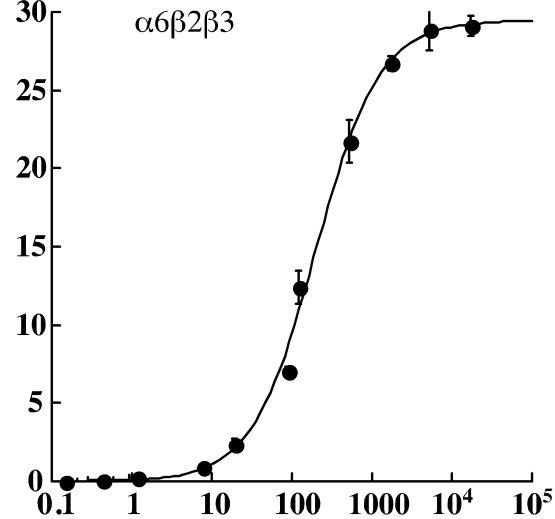
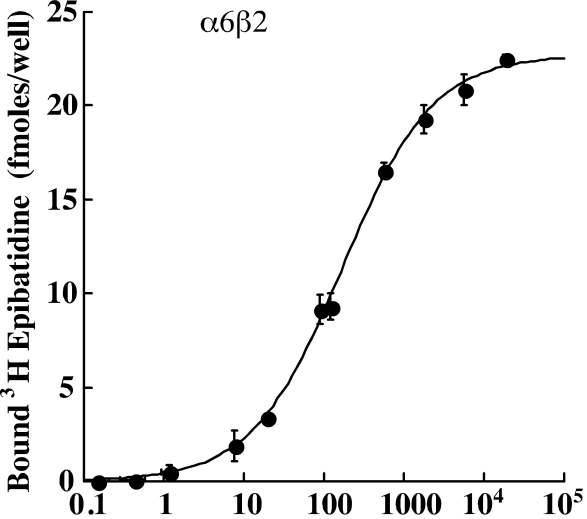


Figure 5



**Figure 6**

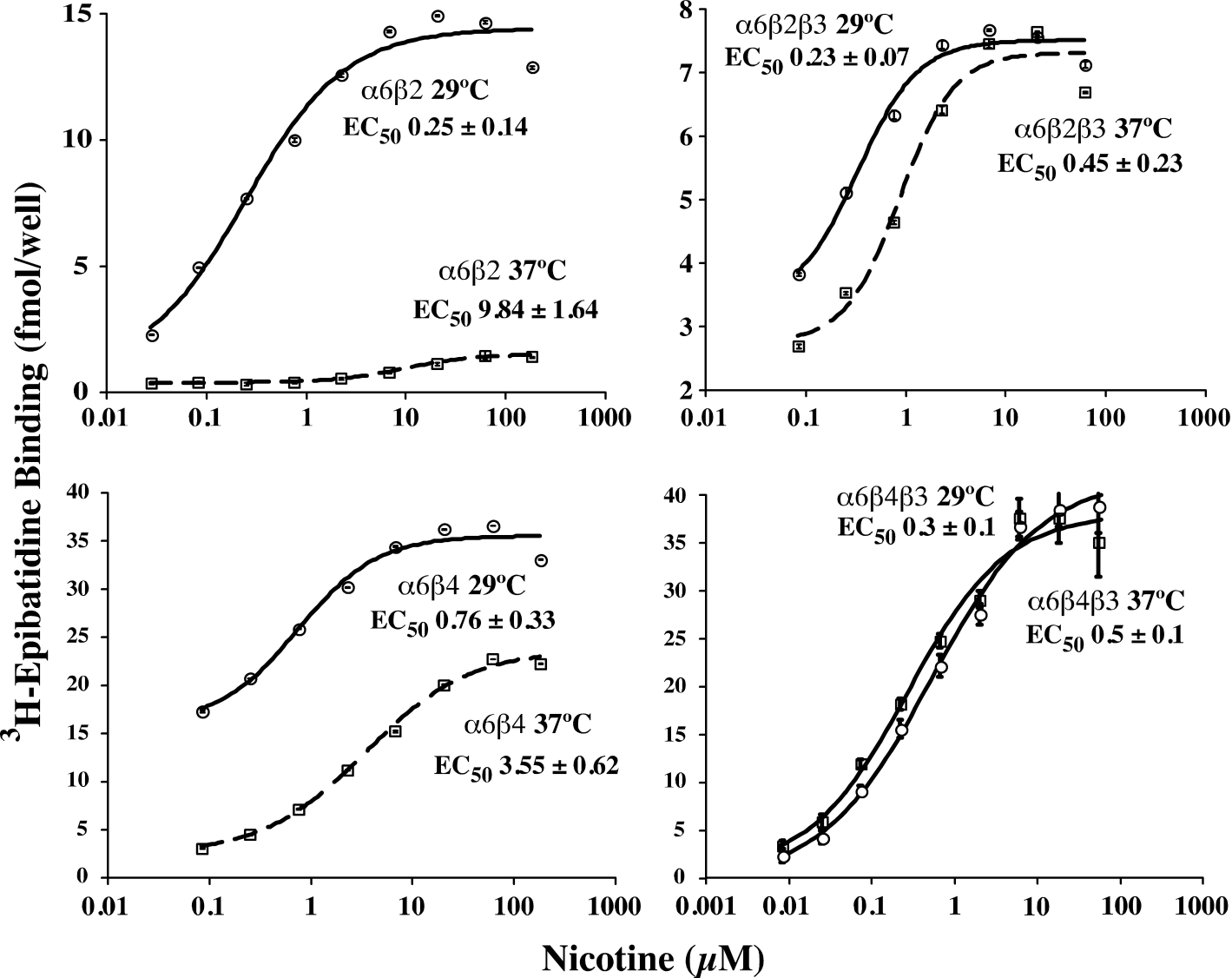


Figure 7