Chronic Nicotine Treatment Up-regulates Human $\alpha_3\beta_2$ but Not $\alpha_3\beta_4$
Acetylcholine Receptors Stably Transfected in Human Embryonic Kidney Cells*

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Human nicotinic acetylcholine receptor (AChR) subtypes $\alpha_3\beta_2$, $\alpha_3\beta_2\alpha_5$, $\alpha_3\beta_4$, and $\alpha_3\beta_4\alpha_5$ were stably expressed in cells derived from the human embryonic kidney cell line 293. $\alpha_3\beta_4$ AChRs were found in prominent 2-μm patches on the cell surface, whereas most $\alpha_3\beta_2$ AChRs were more diffusely distributed. The functional properties of the $\alpha_3$ AChRs in tsA201 cells were characterized by whole cell patch clamp using both acetylcholine and nicotine as agonists. Nicotine was a partial agonist on $\alpha_3\beta_4$ AChRs and nearly a full agonist on $\alpha_3\beta_2\alpha_5$ AChRs. Chronic exposure of cells expressing $\alpha_3\beta_2$ AChRs or $\alpha_3\beta_2\alpha_5$ AChRs to nicotine or carbamylcholine increased their amount up to 24-fold but had no effect on the amount of $\alpha_3\beta_4$ or $\alpha_3\beta_4\alpha_5$ AChRs, i.e. the up-regulation of $\alpha_3$ AChRs depended on the presence of $\beta_2$ but not $\beta_4$ subunits in the AChRs. This was also found to be true of $\alpha_3$ AChRs in the human neuroblastoma SH-SY5Y. In the absence of nicotine, $\alpha_3\beta_2$ AChRs were expressed at much lower levels than $\alpha_3\beta_4$ AChRs, but in the presence of nicotine, the amount of $\alpha_3\beta_2$ AChRs exceeded that of $\alpha_3\beta_4$ AChRs. Up-regulation was seen for both total AChRs and surface AChRs. Up-regulated $\alpha_3\beta_2$ AChRs were functional. The nicotinic antagonists curare and dihydro-β-erythroidine also up-regulated $\alpha_3\beta_2$ AChRs, but only by 3–5-fold. The channel blocker mecamylamine did not cause up-regulation of $\alpha_3\beta_2$ AChRs and inhibited up-regulation by nicotine. Our data suggest that up-regulation of $\alpha_3\beta_2$ AChRs in these lines by nicotine results from both increased subunit assembly and decreased AChR turnover.

Nicotinic acetylcholine receptors (nAChRs) containing $\alpha_3$ subunits are found in autonomic ganglia where they have been found in various combinations with $\beta_2$, $\beta_4$, and $\alpha_5$ subunits (1–3). They have also been found in brain (4), adrenal gland (5), thymus (6), respiratory epithelial cells (7), and keratinocytes (8, 9). In ganglia, $\alpha_3$ AChRs play a postsynaptic role similar to that of muscle AChRs (1, 2). $\alpha_3$ AChRs also have been found in various presynaptic locations in rat brain and implicated in promoting neurotransmitter release (e.g. in dopaminergic and noradrenalinergic pathways) (10–15). We have previously studied the subunit compositions and the pharmacological properties of human $\alpha_3$ AChR subtypes using the Xenopus oocyte expression system and the human peripheral neuroblastoma cell line SH-SY5Y (16–19). We describe here our study of human $\alpha_3$ AChRs stably expressed in a derivative of human embryonic kidney (HEK293) cells. Compared with the Xenopus oocyte expression system, stable mammalian cell lines provide several advantages. 1) We can obtain more reproducible and long lasting levels of AChR expression that are required for detailed single channel studies without the seasonal vagaries of transient expression in Xenopus oocytes. 2) We can better study the effects of chronic exposure to ligands such as nicotine than is convenient in a transient expression system like Xenopus oocytes. 3) Cell lines expressing various AChR subtypes provide a potential for drug discovery by high-throughput screening. Previously, others have reported in article or abstract form permanently transfected cell lines expressing rat $\alpha_3\beta_4$ (20, 21) or human $\alpha_3\beta_2$ (20, 21), $\alpha_3\beta_4$ (23), and $\alpha_3\beta_2\alpha_5$ AChRs (24). This is the first detailed report of a matched set of four cell lines expressing human $\alpha_3\beta_2$, $\alpha_3\beta_2\alpha_5$, $\alpha_3\beta_4$, or $\alpha_3\beta_4\alpha_5$ AChRs.

One important application of human $\alpha_3$ AChR cell lines is to study the effect of chronic nicotine exposure. Addiction to nicotine is characterized by up-regulation of nAChRs in brain (25–27). The number of high affinity nicotine-binding sites in the brains of tobacco smokers and animals chronically given nicotine is increased up to 2-fold (26–30). Most of this increase is in $\alpha_4\beta_2$ AChRs (30), but the amount of increase varies between brain regions and probably involves several AChR types (28, 29). It has been hypothesized that smoking a cigarette results in a rapid bolus of nicotine that activates the mesolimbic dopaminergic system producing pleasure and reward and that nicotine in the smoker’s brain slowly builds to a low steady concentration which causes both reversible desensitization and long term inactivation of some AChR subtypes as well as increases in the amount of some AChR subtypes (19, 31–33). An understanding of the effects of chronic exposure to nicotine on the amount and functional activity of various AChR subtypes might provide better insight into mechanisms of nicotine dependence, tolerance, and withdrawal, as well as the effects of medication with nicotinic drugs.

Chronic exposure to nicotine has been shown to differentially affect both the amount and function of neuronal AChR subtypes. $\alpha_4\beta_2$ AChRs expressed in Xenopus oocytes or a permanently transfected cell line were shown to double in amount.
when chronically exposed to submicromolar concentrations of nicotine (34). These concentrations of nicotine eliminated most α4β2 AChR function due to permanent desensitization (19, 34). On the other hand, a mixture of α3 AChRs expressed by the human neuroblastoma cell line SH-SY5Y increased by 600% in response to chronic exposure to very high concentrations of nicotine (18). Micromolar concentrations of nicotine blocked only a small part of α3 AChR function (19). It has been suggested that some of the behavioral effects of nicotine are likely to depend on the inactivation of both α4β2 and α7 AChRs while leaving α3 AChRs and other subtypes available to respond to endogenous ACh-mediated signaling and to the micromolar boluses of nicotine that occur after inhaling smoke (19, 35). Like mammalian cell lines expressing α4β2 AChRs (34, 36) or α7 AChRs (37), cell lines stably expressing various subtypes of α3 AChRs provide excellent tools for studying the mechanisms and physiological significance of nicotine up-regulation of human α3 AChRs. Here we provide an initial description of these cell lines, including pharmacological and electrophysiological characterization of their AChRs, and study the effects of chronic nicotine exposure on the amount of these AChRs.

**Experimental Procedures**

cDNAs, mAbs, and Antiserum—The cDNAs for human α3, β2, and β4 subunits were cloned in this laboratory and described in our previous report (18). The cDNA for human α5 was kindly provided by Dr. Francesco Clementi (see Ref. 38). The cDNA for the human α3 subunit was subcloned into the selective mammalian expression vector pCDNA.1/Zeo+ (Invitrogen), which carries the Zeocin™ resistance gene. The cDNAs for human β2 and β4 subunits were subcloned into the expression vector pRc/CMV (Invitrogen), which carries the neomycin resistance gene. The cDNA for the human α5 subunit was subcloned into the expression vector pCEP4 (Invitrogen), which carries the hygromycin resistance gene. Monoclonal antibodies to α3 subunits (39), mAb290 to β2 subunits (40), mAb288 to β4 subunits (41, 42), rabbit antiserum 3709 to a synthetic peptide corresponding to amino acids 348–387 of human α3 subunits, and rabbit antiserum 3724 to a synthetic peptide corresponding to amino acids 387–401 of human β2 subunits have been described previously (16). Mouse antiserum Hub4.4 to human β4 subunits was raised against a fusion protein of human β4 subunit extracellular domain and large cytoplasmic domain (43). The fusion protein was constructed in the pGEX-4T-2 vector (Amersham Pharmacia Biotech) and expressed in bacteria. It was affinity purified with a glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column before being used to immunize mice. Hub4.4 does not cross-react with human AChR subunits 4A, β2, or α5 on Western blots (data not shown). Rat antiserum to human α3 subunit was raised against a fusion protein of human α3 subunit extracellular domain and large cytoplasmic domain (43). The fusion protein was constructed in the pET-26b+ vector (Novagen) and expressed in bacteria. It was purified by gel exclusion chromatography before being used to immunize rats. The antiserum to α3 was absorbed with a resin-coupled fusion protein of human α3 AChR subunit extracellular and cytoplasmic domains to remove antibodies to conserved epitopes shared with α3 and other subunits.

**Cell Culture and Stable Transfection of Human tsA201 Cells**—Human tsA201 cells, a derivative of the human embryonic kidney cell line 293 (44), were maintained in Dulbecco’s modified Eagle’s medium (high glucose) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), and 2 μM l-glutamine (Life Technologies, Inc.) in a CO2 (5%) incubator at saturating humidity.

The α3β2 cell lines were established by co-transfecting tsA201 cells with Hu3/pCDNAS.1/Zeo+ and Huβ2/pRc/CMV using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s instructions. The αβ4 cell lines were developed by co-transfecting tsA201 cells with Hu3/pCDNAS.1/Zeo+ and human β4/pRc/CMV following the same procedure. The αβ3β4 cell lines were obtained by transiently transfecting the established αβ3β2 and αβ3β4 cell lines with Hu5/pCEP4. For the cloning of stably transfected cell lines, 72 h after transfection, selection medium containing Geneticin (600 μg/ml, Life Technologies, Inc.) and Zeocin (500 μg/ml, Invitrogen) was used for the αβ3β2 and αβ3β4 cell lines. For the αβ3β2 and αβ3β4 cell lines, the third antibiotic drug hygromycin (200 μg/ml, Boehringer Mannheim) was added to the above selection medium. The initial screening for cell colonies expressing functional αβ3β2 and αβ3β4 AChRs was based on a solid phase radioimmunoassay (RIA) with [3H]epibatidine on mAb210-coated Immulon 4 (Dynatech) microtiter wells. Screening for αβ3β2α5 and αβ3β4α5 cell lines was performed by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 cell lines was performed by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 (36) cell lines. The αβ3β2α5 and αβ3β4α5 cell lines were selected by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 (36) cell lines. The αβ3β2α5 and αβ3β4α5 cell lines were selected by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 (36) cell lines. The αβ3β2α5 and αβ3β4α5 cell lines were selected by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 (36) cell lines. The αβ3β2α5 and αβ3β4α5 cell lines were selected by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 (36) cell lines. The αβ3β2α5 and αβ3β4α5 cell lines were selected by both RIA and Western blot analysis with αβ3β2α5 (35) and αβ3β4α5 (36) cell lines.
media prior to recording. Currents were evoked by application of agonist from one of the two glass perfusion tubes. Prior to application of agonist, the cell was isolated in a continuously flowing control solution from the other tube. Agonists were selected via two 6-way valves in series. Currents were activated by a 2-s application of agonist flowing from the glass tubing attached to a piezo-electric application system that was activated by an isolated pulse stimulator (A-M Systems model 2100). The speed of solution exchange was determined by clamping an open recording electrode at 0 mV and then measuring the change in the solution junction potential when moving from a normal recording solution to one that had been diluted 5-fold with deionized water. The time constant for the change in clamping current during the solution change was approximately 1 ms at the solution flow rates used for activating currents in these cells. Recordings were obtained with an Axopatch 1-D amplifier (Axon Instruments, Inc., Foster City, CA) with the filtered output at 2 kHz and sampled with pClamp 6.0.3 (Axon Instruments) onto a personal computer using a TL-1 DMA interface (Axon Instruments) and a Labmaster A-D converter (Axon Instruments) at 2 kHz. All currents were normalized to the peak current obtained for 300 μACh, and concentration/response curves were fitted to a logistic equation in Origin (Version 4.1; Micral Software, Inc., Northampton, MA). In some cases, the concentration/response relationship would peak and then decline with increasing concentrations of agonist. In the cases where the decline caused an obviously inferior fit, the reduced amplitude responses at higher concentrations were not included in the fit, i.e., for α3β2δ5 both 3 mM ACh and 1 mM nicotine were not included in the fits but are shown in the figures. Representative traces were constructed by opening data files in Axograph 3.55 (Axon Instruments) and exporting data to Canvas 5.0 (Danebo Software, Inc., Miami, FL).

Nicotine Treatment and Electrophysiological Recordings in Xenopus Oocytes—Oocytes were obtained from Xenopus laevis (Nasco). Stage V–VI oocytes were selected and injected with combinations of α3 and β2 or α3 and β4 subunit cRNAs (15 ng of each subunit in a total volume of 55 nl). Currents were measured using a standard two-microelectrode voltage clamp amplifier (Oocyte Clamp OC-725, Warner Instrument Corp., Hamden, CT) as described previously (17). All recordings were digitized using MacLab software and hardware (AD Instruments, Castle Hill, Australia). Data were analyzed using Kaleidagraph (Synergy Software, Reading, PA). For nicotine treatment, the oocytes were incubated overnight at 18 °C in Petri dishes containing the relevant concentration of nicotine. After overnight nicotine treatment, oocytes were placed in dishes of control saline for at least 1 h before electrophysiological recordings.

RESULTS

Expression of AChR Subunit Combinations in Stably Transfected tsA201 Cells—Human embryonic kidney tsA201 cells expressing the AChR subunit combinations α3β2 or α3β4 were initially grown in culture medium containing Zeocin to select for the α3 containing plasmid and neomycin to select for the β2 or β4 containing plasmid. Cell colonies that survived for 3 weeks in the selection medium were tested for AChR expression using a solid phase RIA with [3H]epibatidine as ligand (Fig. 1A). Most (>90%) of the selected colonies contained AChRs that bound [3H]epibatidine. Expression levels varied. Colonies expressing higher levels of AChRs (>200 fmol/mg protein for α3β2 cell lines, and >800 fmol/mg protein for α3β4 cell lines) were recloned by limiting dilution in 96-well plates. Expression of α3 AChRs in α3β2 and α3β4 cell lines was stable with respect to [3H]epibatidine binding for at least 3 months in continuous culture.

Cell lines expressing α3β2δ5 and α3β4δ5 AChRs were established by transfecting lines expressing α3β2 or α3β4 AChRs with a cDNA encoding δ5 and using hygromycin in the culture medium for selection of clones containing δ5 subunits. Expression of δ5 subunits in the α3β2δ5 or α3β4δ5 cell lines was monitored by immunoblots using mAb268 which is specific for denatured δ5 subunits (Fig. 1B).

Our previous studies using the Xenopus oocyte expression sys-
Human α3 AChR Cell Lines

Fig. 2. Sucrose gradient sedimentation analysis of human α3 AChRs expressed in cell lines. AChRs from different cell lines were solubilized with Triton X-100 and were sedimented on 5–20% sucrose gradients. Fractions are numbered from the bottom of the gradients. α3 AChRs were quantitated by [3H]epibatidine binding (4 nM) in a solid phase RIA on mAb210-coated microwells. α3 AChRs isolated from the human neuroblastoma cell line SH-SY5Y were sedimented on a parallel gradient as a size standard for the native AChR.

tem and immunoprecipitation of α3 AChRs incorporating epitope-tagged α5 subunits indicated that co-expression of equal amounts of cRNA for α3, β2 or β4, and α5 subunits resulted in efficient incorporation of α5 subunits into >55% of AChRs (16).

In order to determine how efficiently α5 was incorporated in the α3 AChRs of our cell lines, we performed immunoprecipitation assays with mAb210 (which binds both native α3 and α5 subunits) and an antiserum to bacterially expressed α5 (which binds to both native and denatured α5 subunits) to measure the fraction of αβ2α5 AChRs in the cell line. Data in Fig. 1C show that 49% of α3 AChRs in the cell line contained α5 subunits. In a similar manner we determined that only 14% of the α3 AChRs in the αβ4α5 cell line contained α5 subunits. In order to determine whether inefficient assembly with α5 subunits was an artifact of expression in transfected lines, the fraction of α5-containing AChRs in the human neuroblastoma cell line SH-SY5Y was also measured in the same way. Only about 9% of the α3 AChRs in the native neuronal cell line appeared to contain α5 subunits (Fig. 1C). Thus, it seems that in both transfected cell lines and neuroblastoma cell lines α5 subunits are incorporated into α3 AChRs less efficiently than is the case in Xenopus oocytes.

We also studied the sedimentation behavior of α3 AChRs expressed in the stably transfected cell lines. By comparing their sedimentation properties with those of native α3 AChRs from SH-SY5Y and those of human αβ2 AChRs expressed in Xenopus oocytes, we found that in all four cell lines, the AChR complex (indicated by the binding of [3H]epibatidine) co-sedimented with native α3 AChRs in the 11 S region, which corresponded to fully assembled pentamers (Fig. 2). There was no evidence of partially assembled αβ2 dimers, for example, which would have been expected to form an epibatidine-binding site at their interface (46) but would have sedimented much more slowly than the αβ2α3β2β2 arrangement of five subunits around the central ion channel expected of native AChRs.

Transfected Cells Express Functional AChRs—All transfected cell lines that exhibited binding of [3H]epibatidine responded with currents to applications of nicotine or ACh, although with differing current amplitudes and differing percentages of cells responding (Figs. 3 and 4). The subunit combination αβ4 gave the most robust responses, typically reaching maximal currents of 1–5 nA, with some cells responding with currents as large as 15–20 nA. The EC50 values for activation by ACh and nicotine for αβ4 AChRs were 79 ± 8 and 56 ± 15 μM, respectively. The Hill coefficients were 1.5 and 1.6, respectively. ACh and nicotine had equivalent efficacies in activating αβ4 AChRs in these cells. The currents of the αβ4 cells exhibited a slow decay during agonist application. αβ2 transfected cells had insufficient expression to characterize their functional properties without nicotine-induced up-regulation (see below). Therefore, for functional studies, these cells were incubated for 12 h with 100 μM nicotine followed by a minimum of a 1-h wash in normal culture media prior to recording. All functional studies for β2-containing AChRs expressed in tsA201 cells were preceded by nicotine exposure in this manner. The αβ2 AChRs generally had smaller responses and were undetectable in a greater number of cells. The largest currents for these cells were between 1 and 1.5 nA. The EC50 values for activation by ACh or nicotine were 209 ± 26 and 70 ± 6 μM, respectively. The Hill coefficients were 1.7 and 1.3, respectively. For αβ2 AChRs, nicotine appears to have only 60% efficacy compared with ACh. In stark contrast to the αβ4 AChR responses, the decay of αβ2 currents was extremely rapid and usually complete within 0.5 s, indicating a rapid desensitization rate. The need to expose the β2-containing cell lines to nicotine for functional studies raises the concern that the functional properties of the AChRs might be altered by nicotine exposure. To address this concern we performed a parallel series of experiments in oocytes expressing αβ2 and αβ4 AChRs, which demonstrated that overnight incubation of the oocytes in 100 μM nicotine had no substantial effect on the rates of desensitization (which is also more rapid for αβ2 AChRs expressed in oocytes (16, 49)), EC50 values for activation of the αβ2 or αβ4 AChRs by ACh and nicotine, or the efficacy of nicotine (which also is a partial agonist for AChR responses, the decay of αβ2 currents was extremely rapid and usually complete within 0.5 s, indicating a rapid desensitization rate). The need to expose the β2-containing cell lines to nicotine for functional studies raises the concern that the functional properties of the AChRs might be altered by nicotine exposure. To address this concern we performed a parallel series of experiments in oocytes expressing αβ2 and αβ4 AChRs, which demonstrated that overnight incubation of the oocytes in 100 μM nicotine had no substantial effect on the rates of desensitization (which is also more rapid for αβ2 AChRs expressed in oocytes (16, 49)), EC50 values for activation of the αβ2 or αβ4 AChRs by ACh and nicotine, or the efficacy of nicotine (which also is a partial agonist for Aβ2 AChRs expressed in oocytes (16, 49)) (data not shown).

The EC50 for activation of αβ4α5 AChRs by ACh was 81 ± 15 μM and by nicotine was 42 ± 5 μM. The Hill coefficients were 1.6 and 1.7, respectively. ACh and nicotine had equivalent efficacies. Thus, the pharmacological properties were essentially identical to αβ4 cells. This may not be surprising given that Fig. 1 shows that only 14% of these AChRs incorporated α5 subunits. There was no clear evidence for a dual population of AChRs in the concentration/response relationships. The decay of the currents was indistinguishable from that observed for αβ4 AChR currents. For αβ2 cells transfected with the α5 subunit, the cells were also incubated with 100 μM nicotine to increase AChR expression followed by at least a 1-h wash. The maximal currents were about 1 nA and bore a striking resemblance in time course to the parent αβ2 AChR cell line responses. Despite the 49% incorporation of α5 subunits shown for these AChRs in Fig. 1, there was no evidence of two populations of AChRs greatly differing in their responses. The EC50 for activation by ACh was 121 ± 18 μM and by nicotine was 83 ± 12 μM. The concentration/response relationships had Hill coefficients of 1.6 and 1.3, respectively. The efficacy of nicotine increased in the presence of α5 but remained less than that of
ACh, probably reflecting the somewhat lower incorporation of α5 (49%) in the cells compared with *Xenopus* oocytes (72%) (16). Although the EC50 for activation by ACh was somewhat lower than that found for the α3β2 cells, there were diminishing responses at the saturation end of the concentration/response curves suggestive of agonist-mediated channel blockade that would attenuate the responses leading to an underestimation of the true EC50 value and obscuring the relative efficacies of these agonists. Therefore, it would appear that the α5 subunit does not alter the apparent affinity of this AChR for activation by ACh when compared with the α3β2 AChR, but the α5 subunit might increase the susceptibility to channel blockade by ACh.

**Chronic Treatment with Nicotine Up-regulates α3β2 but Not α3β4 AChRs in Cell Lines**—Chronic exposure of the α3β2 cell line to nicotine increased the amount of α3β2 AChRs up to 24-fold as measured by [3H]epibatidine binding to immunisolated solubilized AChRs (Fig. 5). The EC50 for nicotine-induced up-regulation of α3β2 AChRs was 2 ± 0.3 μM (n = 4). This concentration is 30–40-fold lower than the EC50 for activation of the α3β2 AChRs by nicotine (Fig. 4) but 10-fold higher than the EC50 for up-regulation of α4β2 AChRs (34) or the typical serum concentration of nicotine in a tobacco user (35). Although the maximum effect of nicotine was seen at 1000 μM, α0.2 μM, which is very close to the serum concentration of nicotine for smokers (47), nicotine was able to up-regulate α3β2 AChRs more than 3-fold in 7 h (Fig. 5). At concentrations as high as 1 mM, nicotine did not affect cell proliferation. The up-regulation could be seen as early as 15 min after nicotine (100 μM) exposure and reached a maximum by 8 h. Nicotine also up-regulated α3 AChRs in α3β2α5 cell lines to the same extent (up to 22-fold) as AChRs in the α3β2 cell line (Fig. 5).
Considering that \( \alpha_3 \beta_2 \) AChRs can be up-regulated 24-fold by the same concentration of nicotine (100 \( \mu \)M) and that \( \alpha_3 \beta_2 \alpha_5 \) cells represent 49% of \( \alpha_3 \) AChRs in the cells before (see Fig. 1C) and after chronic exposure to nicotine (data not shown), we conclude that nicotine-induced up-regulation is the same on \( \alpha_3 \beta_2 \) AChRs as on \( \alpha_3 \beta_2 \alpha_5 \) AChRs.

Up-regulation of \( \alpha_3 \beta_2 \) AChRs was also assayed by measuring \( \alpha_3 \) AChRs on the cell surface using 125I-mAb210, which binds to \( \alpha_3 \) and \( \alpha_5 \) subunits. Exposing cells to 10 \( \mu \)M nicotine for 12 h increased surface \( \alpha_3 \beta_2 \) AChRs more than 20-fold, from 7.6 \pm 0.3 fmol/dish to 158.5 \pm 7.6 fmol/dish (n = 3). In contrast, for \( \alpha_3 \beta_4 \) cell lines, the amount of AChRs on the surface was not changed by nicotine (108.7 \pm 1.6 fmol/dish and 102.6 \pm 2.3 fmol/dish, before and after nicotine treatment, n = 3).

AChRs were visualized by labeling fixed cells with mAb210 followed by F-GART (Fig. 6). Without exposure to nicotine, \( \alpha_3 \beta_2 \) AChRs were virtually undetectable on the cell surface, but \( \alpha_3 \beta_2 \) AChRs were detectable inside permeabilized cells. Nicotine added to the culture medium at 10 \( \mu \)M for 12 h dramatically increased \( \alpha_3 \beta_2 \) AChRs on the cell surface and inside the cells. Some clusters of \( \alpha_3 \beta_4 \) AChRs were formed on the cell surface. The average diameter of the clusters was about 1 \( \mu \)m. Most \( \alpha_3 \beta_4 \) AChRs were present on the surface of the cells, and incubation in nicotine did not change their amount or distribution. The clustering of \( \alpha_3 \beta_4 \) AChRs on the surface membrane was striking. Their clusters were bigger (1–3 \( \mu \)m in diameter) and much more frequent than those of \( \alpha_3 \beta_2 \) AChRs.

Up-regulation by nicotine of \( \alpha_3 \beta_2 \) AChRs was studied by immunoblot assay to demonstrate the increase of both \( \alpha_3 \) and \( \beta_2 \) subunits in assembled form. \( \alpha_3 \beta_2 \) AChRs were purified with mAb290-Actigel, which binds specifically to \( \beta_2 \) subunits. Blots of purified \( \alpha_3 \beta_2 \) AChRs were labeled with an antiserum to an...
a3-specific oligopeptide (Fig. 7, lanes 1 and 2). An increase in a3 subunits was detected as early as 15 min after nicotine exposure. The maximum effect was seen after about 8 h. a3b2 AChRs were also immunopurified using mAb210-Actigel, which recognized a3 subunits, and the Western blots labeled with an antiserum to a b2-specific oligopeptide. The amount of

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Immunolabeled on: & mAb290 to b2 & mAb210 to a3 & Anti-b4 serum & mAb210 to a3 \\
\hline
Lane: & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 \\
\hline
Subunit Combinations: & a3 b2 & a3 b2 & a3 b4 & a3 b4 & a3 b3 & a3 b4 & \\
\hline
Nicotine Treatment: & (10\muM, 12 hours) & - & + & - & + & - & \\
\hline
MW(kDa) & 97.4 & - & 66.2 & - & 45.0 & - \\
\hline
\end{tabular}
\caption{AChR Subunit Distribution}
\end{table}

Nicotine up-regulates surface a3b2 AChRs in cell lines. a3 AChRs on the cell surface were visualized by immunofluorescent staining of fixed cells with mAb210 (30 nM) for 1 h at 4 °C followed by F-GART. To visualize total a3AChRs, cells were permeabilized with 0.1% saponin for 15 min before labeling with mAb210. Scale bars: 1, 2, 5, and 10 \textmu m from top to bottom.

Fig. 6. Nicotine up-regulates surface a3b2 AChRs in cell lines. a3 AChRs on the cell surface were visualized by immunofluorescent staining of fixed cells with mAb210 (30 nM) for 1 h at 4 °C followed by F-GART. To visualize total a3AChRs, cells were permeabilized with 0.1% saponin for 15 min before labeling with mAb210. Scale bars: 1, 2, 5, and 10 \textmu m from top to bottom.

Nicotine up-regulates a3b2 but not a3b4 AChRs in the human neuroblastoma cell line SH-SY5Y. SH-SY5Y cells were exposed to 10 \muM nicotine for 48 h. AChRs in the cells were then solubilized with Triton X-100, labeled with [3H]epibatidine (5 nM), and immunoprecipitated with mAb290 (specific for b2 subunits) or antiserum to the bacterially expressed cytoplasmic domain of b4 subunits. Nonspecific precipitation was determined by using in the assay mixture normal rat serum instead of mAb290 or normal mouse serum instead of antiserum to b4. Values represent mean ± S.E. of triplicate determinations. b2 subunits also increased from an early stage of nicotine exposure as well (Fig. 7, lanes 3 and 4). Doublet bands of a3 or b2 subunits were seen on the blots more obviously when cells were treated with nicotine. This probably arises from variable glycosylation of the subunit, because only one band of a3 or b2 was seen on the blots if the purified AChRs were deglycosylated before they were loaded on the SDS-polyacrylamide gel (data not shown).

The up-regulation effect of nicotine was subunit-specific. We
compared the amount of α3β4 AChRs from the cell line before and after nicotine exposure by surface binding assay (Fig. 6), immunoblot assay (Fig. 7, lanes 5–8), and solid phase RIA (Fig. 11A). None of these assays detected a change even 48 h after nicotine was added to the culture medium. α3β4 AChRs, like α3β4 AChRs, were not up-regulated by nicotine (data not shown). In the neuroblastoma cell line SH-SY5Y, there are both α3β2 and α3β4 AChRs (16). It is known that nicotine can up-regulate α3 AChRs in SH-SY5Y cells (18), but it was not clear which subtypes of α3 AChRs were up-regulated. In order to resolve this, we used antibodies specific for β2 or β4 subunits separately to immunoprecipitate α3 AChR subtypes in SH-SY5Y cells. After chronic exposure to nicotine, the amount of β2-containing AChRs in SH-SY5Y cells increased 3-fold, whereas the amount of β4-containing AChRs was not changed (Fig. 8). These observations strongly suggest that β2 subunits play an important role in nicotine-induced up-regulation of α3 AChRs in neurons.

Nicotine-induced Up-regulation of α3β2 AChRs Results from Enhanced Subunit Assembly and Decreased Turnover Rate of AChRs—We tested the possibility that nicotine treatment increased the affinity of α3 AChRs for [3H]epibatidine by comparing the binding to α3β2 AChRs before and after exposure to 100 μM nicotine. The KD value was not changed (107 ± 18 nM versus 126 ± 10 nM, n = 4), which means that nicotine did not increase the affinity of detergent-solubilized immunoprecipitated α3β2 AChRs for [3H]epibatidine. We also excluded the possibility that nicotine exposure increased the affinity of α3β2 AChRs for mAb210 and mAb290 by showing that nicotine-induced up-regulation could also be demonstrated by employing a filter binding assay using solubilized AChRs labeled with [3H]epibatidine that did not require use of mAbs (data not shown). It was clear that the up-regulation resulted from increasing the amount of AChRs rather than from increasing their affinity for both mAbs.

Since up-regulation of α3β2 AChRs started as early as 15 min after the cells were exposed to nicotine, it is reasonable to suggest that the proper folding and assembly of α3 and β2 subunits were enhanced by nicotine. We tested this possibility by probing for β2 subunits on immunoblots of α3β2 cell extracts, both affinity purified by mAb210-Actigel and applied directly as crude extract (Fig. 9, upper panel). Comparing the specific signal for β2 subunits on the blot with and without exposing the cells to nicotine for 30 min, we found only a small increase in the total amount of β2 subunits in the cell, but the amount of β2 subunits that was assembled with α3 subunits greatly increased (more than 5-fold). Since the affinity of α3β2 AChRs for mAb210 was not changed, the large increase in β2 subunits purified by the α3-specific mAb210-Actigel a short time after exposure to nicotine (before the total amount of β2 subunits increased greatly) suggests that the rate of subunit assembly into α3β2 AChRs increased upon nicotine exposure.

To test whether up-regulation of α3β2 AChRs requires protein synthesis, cycloheximide, a protein synthesis inhibitor, was used to block the synthesis of α3 and β2 subunits for 3 h before nicotine was added into the culture medium. After 90 min, the total amount of α3β2 AChRs in the cells was measured by RIA (Fig. 9, lower panel). Compared with control cells, nicotine was still able to up-regulate α3β2 AChRs 6-fold without new protein synthesis. This suggests that at least 25% of the up-regulation in α3β2 AChRs (6-fold up-regulation without increased synthesis versus 24-fold otherwise) is due to enhanced assembly and perhaps the reduced degradation of pre-existing subunits.

Previous studies in our laboratory showed that nicotine was able to decrease the turnover rate of avian α4β2 AChRs stably expressed in mammalian fibroblast cells (34). To see if the up-regulation effect of nicotine for human α3β2 AChRs could also be partially attributed to the stabilization effect of nicotine on α3β2 AChRs in tsA201 cells, we blocked protein synthesis in the cell using cycloheximide and followed the influence of nicotine on α3β2 AChRs in the cells for 24 h (Fig. 10). In the absence of nicotine, the addition of cycloheximide prevented replacement of α3β2 AChRs, and the total amount decreased with a t½ of around 30 h. When nicotine and cycloheximide were added together, there was a rapid initial increase in α3β2 AChRs due to assembly of pre-existing subunits. Western blots like those in Fig. 7 using mAb210 to α3 to isolate assembled AChRs and antisera to β2 to reveal β2 on blots showed about half-maximal assembly by 30 min, about twice this amount by}

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**Fig. 9.** Nicotine enhances subunit assembly of α3β2 AChRs. Upper panel, immunoblots of β2 subunits either affinity purified with mAb210-Actigel to bind AChRs containing α3 subunits or in a crude extract from α3β2 cells were probed with rabbit antiserum to a human β2 subunit synthetic peptide. This showed that nicotine caused an increase of β2 subunits assembled with α3, much more than that of total β2 subunits. Lower panel, protein synthesis in α3β2 cells was blocked with cycloheximide (35 μM) for 3 h before nicotine (100 μM) was added to the culture medium. This showed that nicotine could still cause some up-regulation of α3β2 AChRs in the cells without synthesis of more subunits.

**Fig. 10.** Nicotine decreases the turnover rate of α3β2 AChRs in tsA201 cells. Synthesis of new AChR protein in control or nicotine (100 μM)-treated cells was inhibited with cycloheximide (35 μM) added simultaneously with nicotine. Total AChRs in the cells were measured at 24 h by solid phase RIA with [3H]epibatidine (5 nM). In control cells, the amount of α3β2 AChRs decreased nearly 50% due to turnover, whereas the amount of α3β2 AChRs in nicotine-treated cells remained stable after an initial increase due to enhanced subunit assembly. Each data point represents the mean ± S.E. of triplicate determinations.
Fig. 11. Effects of other nicotinic ligands on α3β2 AChR up-regulation in tsA201 cells. A, α3β2 and α3β4 cells were treated for 12 h with the agonists nicotine or carbamylcholine, the competitive antagonists curare or DHβE, or the channel blocker mecamylamine. The total amount of AChRs in the cells was determined by solid phase RIA of detergent-solubilized AChRs labeled with [3H]epibatidine. All the ligands, except mecamylamine, up-regulated α3β2 AChRs. None of the ligands influenced the amount of α3β4 AChRs. B, a hundred-fold excess of curare, DHβE, or mecamylamine was added to the culture medium of α3β2 cells simultaneously with nicotine. Only mecamylamine significantly inhibited up-regulation of α3β2 AChRs by nicotine. Values represent means ± S.E. of triplicate determinations in solid phase RIA.

3 h, and a slight further increase by 24 h (data not shown). Fig. 10 shows that by 3 h there was a 13-fold increase in α3β2 AChRs. This exceeds the 5-fold increase caused by nicotine after 3 h in cycloheximide shown in Fig. 9, presumably due to turnover of about half of the unassembled α3 and β2 subunits during the 3 h after cycloheximide was added and before nicotine was introduced. Both Fig. 9 and Fig. 10 show that after nicotine is added, and the initial burst of enhanced assembly of α3β2 AChRs is complete, the amounts of α3β2 AChRs remain virtually constant for the duration of the experiments. Thus, in the presence of nicotine the rate of turnover of assembled AChRs is greatly decreased from the t½ of 30 h in control cells to an immeasurably slow t½. Therefore, the 24-fold increase in α3β2 AChRs induced by nicotine in cells in the absence of cycloheximide results both from increased assembly (about 13-fold or about half of the total effect) and from reduced degradation (about the remaining half of the 24-fold increase).

Effects of Other Agonists and Antagonists on Up-regulation of α3β2 AChRs—Nicotine is a tertiary amine that can cross cell membranes, which might permit it to influence AChR subunit maturation or assembly from within a cell. Carbamylcholine is a quaternary amine that cannot penetrate the cells and should act only through AChRs in the cell surface. When carbamylcholine was added to the culture medium of α3β2 cells, the amount of α3β2 AChRs was increased by 2.5-fold within 15 min. The effect of carbamylcholine reached a maximum (25-fold) after about 12 h (Fig. 11A). Thus, in these cells, an agonist acting only on surface AChRs can cause up-regulation equal in extent to that caused by nicotine.

Previous studies with avian α4β2 AChRs expressed in permanently transfected mouse fibroblasts showed that the competitive antagonist curare prevented the 2-fold up-regulation by nicotine (34). Curare and dihydro-β-erythroidine (DHβE) had little or no effect on nicotine-induced up-regulation of α3 AChRs in the human neuroblastoma SH-SYSY (18). Human α4β2 AChRs expressed in permanently transfected human embryonic kidney cells could be up-regulated 15-fold by nicotine (EC 50 = 0.4 μM) and 2–5-fold by curare (EC 50 = 300 μM) or DHβE (EC 50 = 107 μM) (48). In this study, two competitive antagonists, curare and DHβE, were tested for their influence on up-regulation of α3β2 AChRs. Both curare and DHβE induced increases of α3β2 AChRs in tsA201 cells in the absence of nicotine (Fig. 11A), although the effect was not as dramatic as that of nicotine (a maximum of 3-fold for curare and 5-fold for DHβE). When curare and DHβE were co-applied with nicotine at 100-fold molar excess, the up-regulation of α3β2 AChRs by nicotine was not significantly changed (Fig. 11B). It is known from our previous report (34) that at the concentrations applied, both curare and DHβE were very effective at blocking cation flow through α3β2 AChRs. This suggests that at least part of the agonist-induced up-regulation can be explained by mechanisms that do not require cation flow through α3β2 AChRs.

The channel blocker mecamylamine causes up-regulation of AChRs in mouse brains (28) and of avian α4β2 AChRs transfected in mouse fibroblasts (34), but not of human α4β2 AChRs transfected in human embryonic kidney cells (48) or α3 AChRs in the human neuroblastoma SH-SYSY (18). In our study, we found that mecamylamine did not cause up-regulation of α3β2 AChRs in the cells, and it inhibited the up-regulation of α3β2 AChRs by nicotine (Fig. 11, A and B). This phenomenon was observed with another channel blocker, amantadine, as well (data not shown).

DISCUSSION

We established stable cell lines that express four different subtypes of human α3 AChRs: α3β2, α3β2δ5, α3β4, and α3β4δ5. They form functional ion channels in the cell surface. We found that chronic exposure of cells expressing α3β2 AChRs or α3β2δ5 AChRs to nicotine or carbamylcholine up-regulated the amount of AChRs up to 24-fold but had no effect on the amount of α3β4 or α3β4δ5 AChRs, i.e. the up-regulation of α3 AChRs depends on the presence of β2 but not β4 subunits in the AChRs.

Saturation binding curves of α3β2 and α3β4 AChRs with [3H]epibatidine indicated that α3 AChRs stably expressed in tsA201 cells (Kd = 1.26 × 10⁻¹⁰ M for α3β2 AChRs and 4.83 × 10⁻¹⁰ M for α3β4 AChRs) have similar binding affinities as corresponding α3 AChRs expressed in Xenopus oocytes (Kd = 1.2 × 10⁻¹⁰ M for α3β2 and 4.9 × 10⁻⁹ M for α3β4 (16)) or the human neuroblastoma cell line SH-SYSY (Kd = 1.5 × 10⁻¹⁰ M and Kd = 7.4 × 10⁻⁹ M (16)). Sucrose gradient sedimentation analysis indicated that AChRs expressed in the cell lines were consistent with the expected pentameric structure of AChRs.
and revealed no partially assembled AChRs. The expression levels for α3β2 and α3β4 AChRs were different, with α3β2 cell lines producing more AChRs. The different levels of expression between α3β2 and α3β4 in tsA201 cells were also seen in transiently transfected cells by solid phase RIA; thus, this is not an artifact of the particular lines chosen. This suggests that there are intrinsic differences between β2 and β4 subunits in either the efficiencies of synthesis, maturation, or assembly with α3 subunits or that the rate of AChR turnover is different between α3β2 and α3β4 AChRs. We consider the last three possibilities as being more likely because in the absence of protein synthesis nicotine could up-regulate the amount of α3β2 AChRs to a level similar to that found for α3β4 AChRs.

The α3β4 cells expressed high levels of functional AChRs. Responses in the early passages of the cells following recloning were typically 5–10 nA in maximal amplitude at a holding potential of –60 mV. With higher numbers of passages (>15), the responses of the cells were somewhat reduced in amplitude (1–2 nA maximum at –60 mV) but were more than sufficient for functional studies. Concentration/response studies revealed EC50 values for activation by ACh and nicotine of 79 and 55 μM. These values are lower than the values of 163 and 106 μM observed when human α3β4 was expressed in Xenopus oocytes (16). These values are also significantly lower than what has been reported for rat α3β4 AChRs studied in transfected HEK-293 cells, but the order of potency with nicotine > ACh was the same (20). The EC50 values for ACh or nicotine activation of α3β4o5 AChRs (81 or 42 μM, respectively) were not greatly different from α3β4 AChRs. This may reflect the low percentage (14%) of AChRs that contained the α5 subunit in α3α5β4 tsA201 cells.

Even after nicotine-induced up-regulation, α3β2-transfected tsA201 cells responded less robustly than β4-containing AChRs to applications of either ACh or nicotine, having maximum currents of 0.5–2 nA. This may in part reflect the more rapid desensitization of α3β2 AChRs. The EC50 values were 209 and 70 μM for ACh or nicotine, respectively, and nicotine had partial efficacy. α3β2 AChRs expressed in Xenopus oocytes had EC50 values of 28 and 6.8 μM for ACh and nicotine, respectively, and nicotine had an efficacy of 55% (16, 49). The differences in EC50 or response kinetics between the cell lines and Xenopus oocyte expression were not due to the necessity to expose α3β2 AChR lines to nicotine to get substantial expression. We found that overnight exposure of oocytes to 100 μM nicotine followed by 1 h of rinsing did not increase the extent of response, change the kinetics of response, or change the EC50 values for ACh or nicotine on either α3β2 AChRs or α3β4 AChRs (data not shown). Thus, differences between responses of cell lines and oocytes probably reflect a combination of cell biological differences in membrane lipid composition, levels of protein phosphorylation, and other modifications as well as technical differences that permit more rapid and uniform agonist application to small cells than to large oocytes. No published report of the pharmacological properties of human α3β2 AChRs in transfected cells has appeared previously. The additional transfection of the α3β2 tsA201 cells with the α5 subunit resulted in an EC50 for ACh that was slightly lower than that for α3β2 AChRs. The difference could reflect changes in channel gating kinetics but probably is due to agonist-induced channel block brought on by the addition of the α5 subunit to the channel forming region of the AChR causing a premature saturation in the concentration/response relationship. This would cause the appearance of a reduction in the EC50 value, as we believe is the case here, but would not reflect a true change in the affinity of the AChR for ACh. We conclude that although the EC50 values for activation of α3β2 or α3β2α5 AChRs by ACh are different, the difference is probably unrelated to agonist binding. This is consistent with the model proposed previously that the α5 subunit would be located in a position separating the α3 subunits of the α3β2 dimers with the agonist-binding site located between the α3 and β2 subunits (16). In this position, the α5 subunit would not contribute to the agonist-binding site but would contribute to the lining of the channel of the AChR and influence the permeability, conductance, and gating properties of the channel. In contrast to what was observed in AChRs expressed in Xenopus oocytes (16, 49), the current decay of α3 AChRs was not accelerated by the coexpression of the α5 subunit. This might be explained by reduced incorporation of α5 into AChRs expressed in cell lines as compared with oocytes. In the case of α3β2α5 AChRs in the cell lines, 50% incorporation of α5 was sufficient to increase the efficacy of nicotine (relative to ACh) to about 80% as compared with the 100% value observed in oocytes.

Previous studies of human AChRs expressed in Xenopus oocytes indicated that as structural subunits, β2 and β4 make different contributions to the pharmacological as well as ion channel properties of α3 AChRs (16, 17, 50). Chimera constructs between rat β2 and β4 subunits proved that N-terminal extracellular domains of the two subunits are responsible (at least partially) for the different behaviors of α3β2 and α3β4 AChRs upon activation by agonists (51).

Differences in cytoplasmic domains between β2 and β4 subunits may also be important. It has been reported that both β2 and β4 subunits can substitute for β1 subunits in muscle AChRs. Only β4 and not β2, however, can associate with the muscle 43-kDa protein Rapsyn which interacts with the cytoplasmic domain to aggregate AChRs into patches and anchor them to the cytoskeleton (52, 53). We observed that α3β4 AChRs were aggregated into a dense array of patches on the surface of transfected cell lines, whereas α3β2 AChRs were not. It may well be that some Rapsyn-like proteins are expressed in the human embryonic kidney cell line used here and account for the selective aggregation (and thus perhaps stabilization on the cell surface) of α3β4 AChRs. The selective expression of some α3 AChRs at post-synaptic locations and others in patches at peri-synaptic regions in ciliary ganglion neurons (54) may reflect differences in composition of β2, β4, and α5 subunits and in their consequent ability to interact with various AChR-associated proteins and cytoskeletal elements.

We provide further evidence that β2 and β4 subunits play unique roles in α3 AChR subtypes by demonstrating different responses of α3β2 and α3β4 AChRs to chronic nicotine exposure. α3β2 AChRs can be up-regulated by nicotine up to 24-fold in tsA201 cell lines, but no significant change was observed for α3β4 AChRs. The same is also true when other agonists and antagonists were used to regulate the α3 AChRs. We have tested several different clones for each AChR subtype, with both higher and lower levels of expression, to demonstrate that the regulation of α3β2, but not α3β4 AChRs, by nicotinic ligands is a general phenomenon (data not shown). The up-regulation of α3β2 but not α3β4 AChRs in SH-SY5Y cells by nicotine also argues that the subunit dependence of the up-regulation response of α3 AChRs is not an artifact of the transfected cell lines.

Nicotine-induced up-regulation of α3β2 AChRs in tsA201 cells can be detected as early as 15 min after the AChRs have been exposed to the drug. We suggest that the quick increase of α3β2 AChRs results from enhanced assembly of AChRs from existing stocks of subunits in the cell rather than from increased de novo synthesis. This hypothesis is supported by the experiments depicted in Fig. 9. Nicotine treatment during the first 30 min is shown to induce a large increase of assembled
In the peripheral nervous system, most of the AChR subunits appear to be composed of α3 and β4 subunits (1–3), whereas in brain, β2-containing AChRs have been found to be critical for enhancing striatal dopamine release and consequent nicotine addiction (15, 58). Chronic nicotine treatment has been reported to increase dopamine release from the striatum in response to subsequent nicotine challenge (59). According to our study, α3β2 AChRs in transfected cells can be up-regulated more than 3-fold within 7 h by nicotine at concentration as low as 0.2 μM, a serum concentration of nicotine typical for smokers (47). Although at similar nicotine concentrations, α4β2 AChRs were also found to be up-regulated both in rat brain (around 66%) (58), and in a permanently transfected cell line (around 70%) (34), functional analyses demonstrated that virtually all α4β2 AChRs were inactivated whereas 80% of α3 AChRs were still active (19). Thus, we suggest that in a smoker's brain, most of the α4β2 AChRs are permanently desensitized by long term nicotine exposure, but α3β2 AChRs may be both increased in amount and retain function. This may be especially important because, at least in mice, nicotine addiction has been shown to depend on β2 containing AChRs that stimulate dopamine release from striatal neurons (58). On the other hand, scanning different areas of rat brain for changes in [3H]nicotine-binding sites after chronic nicotine exposure showed that not all the nicotine-binding sites were up-regulated, suggesting regional heterogeneity in brain AChR composition (60). Considering that there are AChRs in the brain that are up-regulated by nicotine ligands little (e.g. α7 AChRs (18, 60)) or not at all (e.g. α3β4 AChRs according to our study), it is not surprising that certain regions in brain do not respond to nicotine by up-regulation of their nicotine-binding sites. It is even possible that the same AChR subtype, e.g. α3β2 AChRs, expressed in different neurons may exhibit different responses to nicotine, because α3 AChRs (18) or α4β2 AChRs (34, 48) expressed in different cell types show different degrees of up-regulation. Exploring the mechanism of nicotine-induced up-regulation of human α3 AChRs may provide useful insights on nicotine addiction, as well as on development of nicotinic therapeutic agents for patients with Alzheimer’s disease, Parkinson’s disease, Tourette’s syndrome, schizophrenia, or chronic pain (61).

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