Chronic Nicotine Treatment Up-Regulates \( \alpha_3 \) and \( \alpha_7 \) Acetylcholine Receptor Subtypes Expressed by the Human Neuroblastoma Cell Line SH-SY5Y

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SUMMARY

Chronic exposure to nicotine has been reported to increase the number of nicotinic acetylcholine receptors (AChRs) in brain. The mechanism of up-regulation for the \( \alpha_4\beta_2 \) AChR subtype, which accounts for the majority of high affinity nicotine binding in mammalian brain, has previously been shown to involve a decrease in the rate of \( \alpha_4\beta_2 \) AChR turnover. Here, we report an investigation of the extent and mechanism of nicotine-induced up-regulation of \( \alpha_3 \) AChRs and \( \alpha_7 \) AChR subtypes expressed in the human neuroblastoma cell line SH-SY5Y. Up-regulation of human \( \alpha_3 \) AChRs and \( \alpha_7 \) AChRs, unlike \( \alpha_4\beta_2 \) AChRs, requires much higher nicotine concentrations than are encountered in smokers; the extent of increase of surface AChRs is much less; and the mechanisms of up-regulation are different than with \( \alpha_4\beta_2 \) AChRs. The mechanisms of up-regulation may differ for \( \alpha_3 \) AChRs or \( \alpha_7 \) AChRs. Chronic treatment with nicotine or carbamylcholine, but not \( \beta \)-tubocurarine, mecamylamine, or dihydro-\( \beta \)-erythroidine, induced a 500–600\% increase in the number of \( \alpha_3 \) AChRs but only a 30\% increase in \( \alpha_7 \) AChRs. Chronic nicotine treatment did not increase affinity for nicotine or increase the amount of RNA for \( \alpha_3 \) or \( \alpha_7 \) subunits. The effect of nicotine on up-regulation of \( \alpha_7 \) AChRs was partially blocked by either \( \beta \)-tubocurarine or mecamylamine. The effect of nicotine treatment on the number of \( \alpha_3 \) AChRs was only slightly blocked by the antagonists \( \beta \)-tubocurarine, mecamylamine, or dihydro-\( \beta \)-erythroidine at concentrations that efficiently block \( \alpha_3 \) AChR function. Most of the nicotine-induced increase in \( \alpha_3 \) AChRs was found to be intracellular. The \( \alpha_3 \) AChRs, which accumulate intracellularly, were shown to have been previously exposed on the cell surface by their susceptibility to antigenic modulation. The data suggest that chronic exposure to nicotine may induce a conformation of cell surface \( \alpha_3 \) AChRs that at least in this cell line are consequently internalized but not immediately destroyed.

It is well established that chronic nicotine exposure results in increased binding of \( ^{[3]H}\)nicotine and \( ^{125}\text{I}-\alpha \)-bungarotoxin in brain (1–8). AChRs composed of \( \alpha_4 \) and \( \beta_2 \) subunits have high affinity for nicotine and ACh and account for most of the high affinity nicotine binding in rat brain (9–11). AChRs composed of \( \alpha_3 \) subunits in combination with \( \beta_2 \), \( \beta_4 \), and/or \( \alpha_5 \) subunits have lower affinity for ACh and nicotine than do \( \alpha_4\beta_2 \) AChRs and account for a small amount of high affinity nicotine binding in brain (12). Flores et al. (4) showed that \( \alpha_4\beta_2 \) AChRs are increased in the cortex of rats chronically treated with nicotine. In addition, Collins et al. (5, 6) reported that chronic exposure to nicotine or the antagonist mecamylamine increased mouse brain \( ^{[3]H}\)nicotine binding in numerous regions to various extents without increasing the levels of \( \alpha_4 \) or \( \beta_2 \) AChR subunit mRNAs. We found that chronic treatment of Xenopus laevis oocytes expressing \( \alpha_4\beta_2 \) AChRs or a mouse fibroblast cell line permanently transfected with chicken \( \alpha_4\beta_2 \) AChRs with nicotine or mecamylamine caused a \( \sim \)2-fold increase in \( \alpha_4\beta_2 \) AChRs (13). The nicotine concentration dependence, time course, and extent of \( \alpha_4\beta_2 \) AChR up-regulation are similar to those reported for \( \alpha_4\beta_2 \) AChRs in mammalian brains. The nicotine-induced increase in \( \alpha_4\beta_2 \) AChRs is due to a decrease in the rate of \( \alpha_4\beta_2 \) AChR turnover (13). This induction mechanism does not seem to require cation flow through \( \alpha_4\beta_2 \) AChRs because the channel blocker mecamylamine causes up-regulation (6, 13). Nicotine and mecamylamine are synergistic in causing up-regulation (6, 13) because mecamylamine preferentially blocks open channels and nicotine is an agonist, so together they are more effective at accumulating the inactive conformation of \( \alpha_4\beta_2 \) AChR.

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ABBREVIATIONS: \( \alpha \)-Bgt, \( \alpha \)-bungarotoxin; AChR, acetylcholine receptor; mAb, monoclonal antibody; DH\( \beta \)-E, dihydro-\( \beta \)-erythroidine; EGTA, ethylene glycol bis(\( \alpha \)-aminoethyl ether)\( N,N,N',N' \)-tetraacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SSPE, standard saline/phosphate/EDTA; MOPS, 3-(N-morpholino)propanesulfonic acid.
which is turned over more slowly (13). Neuronal AChRs that
bind αβgt have been found to contain α7, α8, or α9 subunits
(14–16). α7 AChRs are the predominant form of αβgt binding
protein in brain (14); they have higher affinity for nicotine
than for ACh but much lower affinity for nicotine than do
α4β2 AChRs (17). Marks et al. (7) reported that chronic
intravenous infusion of mice with nicotine elicited an in-
crease in brain 125I-αBgt binding. The extent and duration of
nicotine-induced up-regulation of 125I-αBgt binding in rat
brains were less than the increase in [3H]nicotine binding (7).
More recently, Barrantes et al. (18) reported that chronic
nicotine treatment of hippocampal neurons with nicotine
elicits a 40% increase in the number of 125I-αBgt binding
sites. These results indicate that the up-regulation of α7-
containing AChRs requires a higher dose of nicotine and a
longer exposure time than does up-regulation of α4β2
AChRs.

After we and others (13, 19, 20) determined that chronic
exposure to nicotine causes up-regulation of the α4β2 AChR
subtype by reducing turnover, it seemed important to deter-
mine whether other AChR subtypes were similarly regu-
lated. Differences in the effects of chronic nicotine exposure
on various AChR subtypes might help to account for varia-
tions in the extent of nicotine-induced up-regulation of [3H]nicotine
binding in various brain regions (5) and for complexities in functional effects of chronic exposure to nicotine
in smokers or chronic exposure to other nicotinic ago-
nists that might be used for therapeutic purposes. The
human neuroblastoma cell line SH-SY5Y, like chick ciliary
ganglion neurons (21), expresses both α3 AChRs and α7
AChRs (22, 23). These cells resemble human fetal sympa-
thetic neurons grown in primary culture and express mRNAs
for α3, α5, α7, β2, and β4 subunits (24). They express α3-
containing AChRs of at least two subtypes, half of which
contain β2 subunits (23), and which contain some mixture of
α3β2, α3β2α5, α3β4, α3β4α5, and α3β2β4α5 subtypes. They
also express α7 AChRs that are either homomers of α7 sub-
units or contain α7 assembled with other, unidentified sub-
units (22, 24, 25). Experiments were undertaken to investi-
gate whether chronic exposure to nicotine of these cells
induces up-regulation of α3 AChRs and α7 AChRs and the
mechanisms that may be involved in this up-regulation.

Materials and Methods

Cell cultures. SH-SY5Y cells were initially provided by June
Biedler and Barbara Spengler of the Sloan Kettering Institute for
Cancer Research (26). Cultures were grown in a 1:1 mixture of Ham’s
F12 medium and Eagle’s minimal essential medium containing 1 ×
10−4 M nonessential amino acids, supplemented with 10% fetal bo-
vine serum, in a 95% air/5% CO2 humidified incubator at 37°. pH of
the L-nicotine (Sigma) solutions was adjusted with 10 N NaOH before
being added to media. The cell monolayers were washed with PBS
saline, scraped, pelleted in a microfuge at 4°, and stored at −80°
until use.

mAbs. mAb210 was initially raised to the main immunogenic
region on the extracellular surface of mammalian muscle α1 sub-
units (27) and was shown to cross-react with human α3 and α5
subunits (23). mAb306 was prepared using as antigen a mixture of
affinity-purified native and denatured αβgt-binding AChRs from the
brains of chickens and rats (14) and was found to cross-react with
human α7 subunits in the SH-SY5Y cell line (22).

Labeling reagents. L-[3H]nicotine (72 Ci/mmol) and [3H]epipha-
tidine (56.6 Ci/mmol) were obtained from New England Nuclear Re-

search Products (Boston, MA) (72 Ci/mmol). mAbs 210 and 306 were labeled with 125I to a specific activity of 7 × 1067 cpm/mol. αBgt was labeled with 125I to a specific activity of 1.07 × 1018 cpm/mol.

Northern blot. Total cellular RNA was isolated according to the
method of Chomczynski and Sacchi (28) from SH-SY5Y cells that had
been treated with or without 1 × 10−3 M nicotine for 4 days. Subse-
sequently, poly(A)1-tailed mRNA was isolated, and 3 μg was elec-
rophoresed for 4 hr at 90 V in a 1% agarose gel containing 1.1 M
formaldehyde, 0.02 M MOPS, 0.05 M sodium acetate buffer, pH 8.0,
and 0.01 M EDTA. The gels were rinsed in water treated with 0.1% (v/v)
diethylycarboxylate and then soaked for 45 min in 10× SSPE
(1× SSPE contains 180 mM NaCl, 10 mM sodium phosphate buffer,
pH 7.4, 1 mM EDTA). RNA was vacuum transferred to a Nytran
membrane (Schleicher & Schuell, Keene, NH) and UV cross-linked.
Hybridization was performed by using a random-primed, 32P-dATP-
labeled, human α3 or α7 subunit cDNA fragment or 32P-UTP-labeled
human β-actin RNA fragment in 40% formamide, 5× Denhardt’s
solution (1× Denhardt’s solution contains 0.02% Ficoll, 0.02% poly-
vinylylpyrrolidone, 0.02% bovine serum albumin), 0.5% SDS, 5×
SSPE, and 0.15 mg/ml denatured salmon sperm DNA and incubated
overnight at 42° for cDNA probes and at 60° for RNA probes. Human
β-actin was used as a heterologous probe to determine the amount of
human β-actin RNA expressed in the SH-SY5Y cell line by allowing
for normalization of α3 and α7 mRNA signals within each lane.
Membranes were washed at 50° in 1× SSPE/0.1% SDS and exposed
to Kodak XAR-5 film at −70°. Scanned images were quantified using
NIH Image 1.54 software.

Cell surface binding. Confluent cells in 60-mm dishes were
treated with or without 1 × 10−7 M nicotine for 4 days. To label α3
AChRs, cells were incubated with 1 ml of medium containing 1 ×
10−7 M 125I-mAb210 for overnight at 4°. Nonspecific binding was
determined in the presence of 1 × 10−6 M unlabeled mAb210. α7
AChRs were similarly labeled using 1 × 10−7 M 125I-αBgt overnight
at 4°. Nonspecific binding was determined in the presence of 1 ×
10−6 M unlabeled αBgt. In both cases, cells were washed three times
with 1 ml of PBS, detached, pelleted, and resuspended before
g- counting.

Membrane fraction binding assays. Cells were grown just as
for cell surface binding experiments and then harvested in PBS.
Cells were lysed by incubation for 1 hr in 4° hypotonic buffer (5 mM
Tris-HCl, pH 7.5) followed by homogenization (34). Centrifugation
at 40,000 × g for 20 min yielded a crude membrane pellet, which was
resuspended in PBS. Labeling with 1 × 10−7 M 125I-mAb210 or
125I-αBgt was conducted overnight at 4° with gentle shaking. Un-
bound labels were removed by pelleting followed by three washes in
PBS and pelleting before resuspension and γ-counting. Nonspecific
binding was determined in the presence of 1 × 10−6 M unlabeled
ligand.

Immunosolated AChR binding assays. For the [3H]nicotine
binding assay, α3 AChRs from SH-SY5Y were solubilized in 5 vol-
umes of lysis buffer (containing 2% Triton X-100, 50 mM NaCl, 50 mM
sodium phosphate buffer, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM
phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 5 mM iodoac-
etamide) through brief vortexing followed by 20 min of gentle rota-
tion at 4° and then a 20-min centrifugation in a microfuge at 4°. The
α3 AChRs were immunoisolated by incubating overnight at 4° the
solubilized AChR with mAb210-coated Immulon 4 microwells (Dyn-
atech Labs, Chantilly, VA). The microwells were then washed three
times, and 100 μl of 2 × 10−6 M [3H]nicotine in 0.5% Triton X-100
PBS buffer was added and incubated 1 hr at 4°. After three rapid
washes, bound [3H]nicotine was removed using sample buffer (2.5% SDS,
5% β-mercaptoethanol) and measured using a scintillation
counter. For the 125I-αBgt binding assay, solubilized AChRs were
incubated with mAb306-coated Immulon 4 microwells overnight
at 4°. The microwells were then rinsed and incubated with 100 μl of 5 ×
10−6 M 125I-αBgt in 0.5% Triton X-100 PBS buffer, pH 7.5. After three
washes, bound 125I-αBgt was measured using a γ-counter. Nonspe-
cific binding was measured using wells lacking mAb.
Electrophysiological recordings from *X. laevis* oocytes injected with 5 ng each of cRNAs for human AChR subunits in the combinations a5β2, a5β4, or a5β2β4α5 were made as previously described (12, 23). Data were collected 3 days after injection using oocytes voltage-clamped at –50 mV.

**Antigenic modulation.** Three 60-mm dishes of confluent SH-SY5Y cells for each condition were grown for 3 days with or without 1 × 10⁻⁷ M nicotine and/or 1 × 10⁻³ M mAb210. Cells from each dish were harvested separately and then lysed by incubation for 1 hr in 5 mM Tris-HCl buffer, pH 7.5, followed by homogenization for 10 sec using a Polytron. Membrane fragments were pelleted by centrifugation for 20 min at 40,000 × g and then resuspended in 200 μl of PBS. After incubation with 1 × 10⁻⁸ M [³H]epibatidine for 2 hr at 4° with gentle agitation, the membranes were washed three times by filtration on Whatman GF/C filters with 3 ml of PBS. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled epibatidine. Bound [³H]epibatidine was measured by scintillation counting.

**Statistical analysis.** Two-tailed *t* tests were used.

**Results**

Chronic exposure to nicotine of cultured SH-SY5Y cells caused a 575% increase in the amount of immunoisolated α3 AChRs but only a 30% increase in the amount of immunoisolated α7 AChRs (Fig. 1). mAb210, which binds to both α3 and α5 subunits (23), and mAb306, which binds to α7 subunits (22), were used to tether AChRs that had been solubilized with Triton X-100. Measurement of total α3 AChRs by binding ¹²⁵I-mAb210 or total α7 AChRs by binding ¹²⁵I-oBgt to cell membrane fragments gave results similar to those obtained using immunoisolated solubilized AChR subtypes (data not shown). The half-maximally effective concentrations of nicotine for up-regulation (EC₅₀), assuming that 1 × 10⁻³ M nicotine gave the maximum response, were 1 × 10⁻⁴ M for α3 AChRs and 6.5 × 10⁻⁵ M for α7 AChRs. This contrasts with the much lower EC₅₀ value for up-regulation of αβ2 AChRs of 2 × 10⁻⁷ M that we had previously observed (13). At a concentration of 1 × 10⁻³ M nicotine, the cells started to detach from the dishes, so 1 × 10⁻³ M was the highest concentration used. Unlike the case with αβ2 AChRs (13), mecamylamine at a concentration of 1 × 10⁻³ M did not cause up-regulation of either α3 or α7 AChRs.

Kinetics of up-regulation of α3 AChRs differed from those of α7 AChRs (Fig. 2). The increase of α3 AChRs was seen as early as 5 hr after nicotine exposure, and the maximum effect was seen after 3 days. Up-regulation of α7 AChRs was seen after an 8-hr exposure and was complete within 24 hr.

The up-regulation resulted from an increase in the amount of AChR rather than from an increase in the affinity of α3 AChRs for nicotine. This was shown by Scatchard plots of [³H]nicotine binding to control and chronically nicotine-treated SH-SY5Y cells (Fig. 3). There are two populations of α3 AChRs in the SH-SY5Y cell line that differ in affinity for nicotine. The affinity of each population for nicotine was not significantly changed after up-regulation.

Northern analysis was used to determine whether the increased AChRs were due to an increased RNA level. Poly(A)⁺-tailed mRNA was isolated from cells treated with or without 1 × 10⁻³ M nicotine for 4 days. Nicotine treatment did not up-regulate the steady state amounts of mRNA for α3 or α7 subunits in SH-SY5Y cells (Fig. 4). The average values from two independent experiments revealed ratios of nicotine-treated to control values of 1.0 for α3 mRNA and 0.84 for α7 mRNA. These results indicated that nicotine up-regulates both α3 and α7 AChRs via post-transcriptional mechanisms.

Most of the α3 AChRs induced by nicotine were found in an intracellular compartment (Fig. 5). To test whether this might be due to nicotine acting inside the cells to facilitate α3 AChR synthesis or assembly, up-regulation by carbamylcholine was also tested. Although nicotine is a tertiary amine that can cross cell membranes, carbamylcholine is a quaternary amine that cannot penetrate the cells to act on AChR synthesis. Carbamylcholine also caused an increase in intracellular α3 AChRs (Fig. 5); this suggests that either carbamylcholine mediated an increase in internal α3 AChRs through mechanisms subsequent to cation flow through α3 AChRs that it stimulated on the surface or that the internal α3 AChRs had been on the surface to interact with carbamylcholine at some time during the 4-day incubation. Surface α3 AChRs were quantified by binding of ¹²⁵I-oBgt to intact cells, and total α3 AChRs were quantified by binding to membrane fragments. Measurements of total α3 AChRs by binding of [³H]nicotine to detergent-solubilized α3 AChRs immunoisolated on mAb210-coated microwells gave similar results (data not shown). Chronic treatment with either nicotine or carbamylcholine caused a > 300% increase in the total amount of α3 AChRs in SY-SY5Y cells but only about a 30% increase in α3 AChRs on the cell surface.

Chronic treatment of SH-SY5Y cells with a high concen-
tration (1 × 10⁻³ M) of the competitive antagonist d-tubocurarine or DHβE or with the noncompetitive open channel blocker mecamylamine did not change the number of α3 or α7 AChRs (data not shown). Up-regulation of α3 AChRs by 5 × 10⁻⁴ M nicotine was not inhibited by a 2 × 10⁻⁴ M concentration of any of these antagonists (data not shown). Even if we decreased the concentration of nicotine to 1 × 10⁻⁵ M and increased the concentration of the antagonists to 1 × 10⁻³ M to provide a 100-fold molar excess of antagonist, the up-regulation of α3 AChRs was not significantly blocked (Fig. 6). A 100-fold molar excess of the antagonists was very effective at blocking cation flow through α3 AChRs (Fig. 6). This suggests that up-regulation of α3 AChRs induced by agonists does not require cation flow through these AChRs.

Evidence that the internalized α3 AChRs induced by agonists had been on the cell surface at some point during the 4 days of incubation with agonist was provided by showing that nicotine-induced α3 AChRs on SH-SY5Y cells were susceptible to antigenic modulation by mAb210 (Fig. 7). It is well known that both antibodies to the main immunogenic region on the extracellular surface of muscle AChR α1 subunits (e.g., mAb210) and autoantibodies to muscle AChRs that are from patients with myasthenia gravis cause down-regulation of AChRs via the process of antigenic modulation (35). This
involves cross-linking of AChRs by antibodies, which facilitates their endocytosis and lysosomal destruction. Because mAbs cannot cross cell membranes, demonstration that mAb210 can prevent most of the nicotine-induced increase in α3 AChRs shows that those α3 AChRs must have been exposed on the surface membrane, where they were accessible to binding by the mAb. The observation that mAb210 did not significantly reduce the amount of α3 AChRs in cells that were not exposed to nicotine may result from the normally very low density of α3 AChRs in these cells, which may make it difficult to cross-link these AChRs into aggregates sufficiently large to speed endocytosis.

The rate of degradation of α3 AChRs in SH-SY5Y cells was assayed using the same approach that we had used to detect a nicotine-induced decrease in the rate of degradation in α4β2 AChRs permanently transfected into mouse fibroblasts (13) (Fig. 8). This method involves the use of cycloheximide to prevent the synthesis of new AChRs, followed by measurement of the rate of loss of existing AChRs in the presence or absence of nicotine. Unlike what we had observed with α4β2 AChRs (13), the presence of nicotine did not slow the rate of loss of α3 AChRs (Fig. 8). This was unexpected because it seemed reasonable to suppose that the nicotine-induced increase in internalized α3 AChRs that we had observed would be reflected in a decrease in turnover rate. However, it may be that prevention of lysosomal destruction of the internalized α3 AChRs that accumulate in the presence of nicotine depends on the continued synthesis of a protein, with the result that when protein synthesis is blocked with cycloheximide, not only the synthesis of new α3 AChRs but inhibition of the destruction of the internalized α3 AChRs is prevented.

The slight extent of up-regulation of α7 AChRs induced by nicotine was substantially blocked by both curare and mecamylamine (Fig. 9). The differences in extent and antagonist sensitivity of up-regulation of α7 AChRs compared with those of α3 AChRs suggest that different mechanisms may be involved in nicotine-induced up-regulation of these two AChR subtypes.

**Discussion**

We found that human α3 and α7 AChRs are up-regulated by chronic exposure to nicotine but only at concentrations of nicotine much higher than those required for up-regulation of α4β2 AChRs (13). The maximum extent of up-regulation was least for α7 AChRs, intermediate for α4β2 AChRs, and highest for α3 AChRs. However, the large amounts of nicotine-induced α3 AChRs are found intracellularly, where they would not be functional; this is summarized in Table 1. In normal rat brains, there seems to be approximately equal amounts of α7 and α4β2 AChRs but much fewer α3 AChRs. α4β2 AChRs are up-regulated with an EC50 value of $2 \times 10^{-7}$ M nicotine, which is also a serum concentration that is typical of tobacco users (29), whereas up-regulation of α3 and α7 AChRs requires nicotine concentrations of ≥400-fold higher. Both α3 and α7 AChRs require much higher nicotine concentrations for activation than do α4β2 AChRs, and the equilibrium binding affinity for nicotine of their presumably desensitized states is much lower than that of α4β2 AChRs. There is no precise correlation between $K_d$ values for binding or EC50 values for activation and EC50 values for up-regulation.
Antagonists of α3 AChR up-regulation in SH-SY5Y Cells

Fig. 6. Antagonists at 100-fold molar excess do not block nicotine-induced up-regulation of α3 AChRs, even though nicotine binding is substantially reduced and current flow is virtually completely blocked. A, α3 AChR up-regulation. SH-SY5Y cells were treated with nicotine with or without antagonists at the indicated concentrations for 4 days. Cells were then harvested, and AChRs were solubilized before solid-phase radioimmunonassay. Each value represents the mean of three dishes. Up-regulation was not significantly blocked (p > 0.05). B, Inhibition of [3H]nicotine binding. α3 AChRs solubilized from SH-SY5Y cells were immunoisolated on mAb210-coated microwells and then incubated with 2 × 10⁻⁸ M [3H]nicotine with or without a 100-fold molar excess of antagonists for 1 hr at 4°C. After three rapid washes, bound [3H]nicotine was measured by scintillation counting. Each value is the mean of duplicate experiments (range is shown). Binding of [3H]nicotine to membrane fragments. Bars, mean of determinations (range is shown). Binding of [3H]nicotine to membrane fragments. Bars, mean of determinations (range is shown).

Inhibition of [3H]nicotine binding to α3 AChRs

Fig. 7. Nicotine-induced α3 AChRs are susceptible to antigenic modulation by mAb210. SH-SY5Y cells were cultured for 3 days with or without 1 × 10⁻³ M nicotine and 1 × 10⁻⁷ M mAb210. Then, their total content of α3 AChRs was determined by measurement of binding of [3H]epibatidine to membrane fragments. Bars, mean of determinations on triplicate 60-mm culture dishes. The loss of most of the nicotine-induced increase in α3 AChRs as a result of the presence of mAb210 suggests that these α3 AChRs appeared on the cell surface and were then cross-linked by the mAbs into aggregates that were endocytosed and destroyed in lysosomes.

**Nicotine Receptor Up-Regulation**

Nicotine-induced α3 AChRs are susceptible to antigenic modulation by mAb210. SH-SY5Y cells were cultured for 3 days with or without 1 × 10⁻³ M nicotine and 1 × 10⁻⁷ M mAb210. Then, their total content of α3 AChRs was determined by measurement of binding of [3H]epibatidine to membrane fragments. Bars, mean of determinations on triplicate 60-mm culture dishes. The loss of most of the nicotine-induced increase in α3 AChRs as a result of the presence of mAb210 suggests that these α3 AChRs appeared on the cell surface and were then cross-linked by the mAbs into aggregates that were endocytosed and destroyed in lysosomes.

regulation argues strongly that for those subtypes, AChR activation is not required for up-regulation. In the case of α7 AChRs, both curare and mecamylamine seemed to block the small amount of nicotine-induced up-regulation, so in the case of this subtype, up-regulation may depend on AChR activation.

Total α3 AChRs in SH-SY5Y cells could be up-regulated to a larger extent (575%) (Fig. 1) than the 100% that is typical of chick α4β2 AChRs (13) or mammalian brain α4β2 AChRs (4), but the extent of surface up-regulation of α3 AChRs was only 30–40% (Fig. 5). Thus, if the effects in SH-SY5Y cells reflect the effects of chronic nicotine exposure on human brain and ganglia, one might expect little nicotine-induced increase in the number of surface α3 AChRs or α7 AChRs in contrast with a doubling of surface α4β2 AChRs. However, because nicotine can also induce functional desensitization of AChRs and the extent and reversibility of this desensitization may vary with AChR subtype, the net relative sensitivity of various AChR subtypes after chronic exposure to nicotine may not be reflected even in the relative amounts of various AChR subtypes in cell surfaces.

The agonist-induced increase in [3H]nicotine binding to immunoisolated AChRs results from an increase in the amount of AChRs rather than from an increase in the affinity of the AChRs for nicotine (Fig. 3). There are two classes of binding sites for [3H]nicotine in SH-SY5Y cells (K_d = 0.5 nM, K_d = 17 nM) (Fig. 3). These results are similar to the results reported by Lukas et al. (24). These two binding affinities may reflect the relative amounts of α3 AChR subtypes present among the possible α3β2, α3β2α5, α3β4, α3β4α5, and α3β2β4α5 subunit combinations (23). For example, we also found that half of the α3 AChRs in SH-SY5Y cells contain β2 subunits and that this half of the AChRs is associated with much higher affinity for epibatidine (23). The effect of nicotine-induced regulation of α3 AChRs in SH-SY5Y cells differs from that in chick ciliary ganglion neurons (21). The α3 AChRs expressed in chick ciliary ganglion neurons are reduced 30% by chronic exposure of cultures to carbamylcholine; this might be accounted for by differences in neuronal
cell types, species, or \(\alpha 3\) AChR subtypes. In chicken ciliary ganglion neurons, 80% of \(\alpha 3\) AChRs have the subunit composition \(\alpha 3\alpha 5\beta 4\) (30), but in SH-SY5Y cells, \(\approx 56\%\) of the \(\alpha 3\) AChRs contain \(\beta 2\) subunits (23).

\(\alpha 7\) AChRs were also up-regulated by nicotine (Fig. 1) but only by very high concentrations of nicotine and to a lesser degree than were \(\alpha 4\beta 2\) AChRs (13). Up-regulation of \(\alpha 7\) AChRs reached its maximum within 24 hr (Fig. 2), mimicking the up-regulation observed in vivo (7). In mice, higher nicotine doses are required to elicit increases in brain \(^{125}\text{I}\)-\(\alpha\)Bgt sites than are necessary to increase \(^{3}\text{H}\)nicotine binding sites, and the amounts of \(^{125}\text{I}\)-\(\alpha\)Bgt sites change more rapidly (7, 31). The relatively small magnitude of up-regulation for \(\alpha 7\) is reminiscent of the small changes in \(^{125}\text{I}\)-\(\alpha\)Bgt binding observed after in vivo administration of nicotine (7, 30). Brain \(^{125}\text{I}\)-\(\alpha\)Bgt binding sites are up-regulated only by higher doses of nicotine (32, 33). This also reflected the lower level of up-regulation that resulted from chronic nicotine exposure of \(\alpha\)Bgt binding sites compared with \(^{3}\text{H}\)nicotine binding sites that was observed in rat brain by Marks et al. (7). Up-regulation of muscle-type AChRs expressed by TE671 cells required \(1 \times 10^{-2}\) M nicotine for significant up-regulation to occur (34, 36). Thus, the effective nicotine concentration reflects the sensitivity of the particular nicotinic AChR subtype to this agonist.

Table 1

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Activation (E_{50})</th>
<th>Binding (K_d)</th>
<th>Up-regulation</th>
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<tr>
<td>(\alpha 4\beta 2) AChRs</td>
<td>0.35 (a)</td>
<td>0.004 (a)</td>
<td>0.21 (a)</td>
</tr>
<tr>
<td>(\alpha 3) AChRs</td>
<td>30 (b)</td>
<td>0.02</td>
<td>100</td>
</tr>
<tr>
<td>(\alpha 7) AChRs</td>
<td>40 (c)</td>
<td>1.3 (c)</td>
<td>65</td>
</tr>
</tbody>
</table>

\(a\) ref. 13
\(b\) ref. 24
\(c\) ref. 22

The failure of nicotine to up-regulate transcription of \(\alpha 3\) and \(\alpha 7\) subunit mRNA (Fig. 4) while up-regulating the amount of \(\alpha 3\) and \(\alpha 7\) AChRs in SH-SY5Y cells is consistent with similar results observed in brain with \(\alpha 4\beta 2\) AChRs (5) and suggests that nicotine also up-regulates \(\alpha 3\) and \(\alpha 7\) AChRs via post-transcriptional mechanisms. This is also reminiscent of results with \(\alpha 4\beta 2\) AChRs in transfected cells (13, 37).

In the case of \(\alpha 4\beta 2\) AChRs, the competitive antagonist curare blocks nicotine-induced up-regulation of cell surface \(\alpha 4\beta 2\) AChRs, the channel blocker mecamylamine causes up-regulation and is synergistic with nicotine in causing up-regulation (13). This was interpreted to mean that agonists and mecamylamine induced a conformation, probably a desensitized conformation, of the \(\alpha 4\beta 2\) AChRs that is turned over more slowly and that up-regulation does not require cation flow through the AChR.

The mechanism of \(\alpha 3\) AChR up-regulation, although similarly post-transcriptional and also apparently not requiring cation flow through the AChRs (Fig. 6), is different from that of \(\alpha 4\beta 2\) AChRs, especially in accumulating a large excess of...
internal α3 AChRs in response to chronic exposure to high concentrations of nicotine. Unlike the effect on αβ2 AChRs (13), the channel blocker mecamylamine itself does not cause up-regulation of α3 AChRs, and it had no synergistic effect with nicotine in causing up-regulation (Fig. 6). This suggests that mecamylamine does not induce the conformation of α3 AChRs required for up-regulation, but neither does it seem to prevent nicotine from inducing this conformation. The observation that after 3 days in the presence of nicotine most of the α3 AChRs induced by agonists were intracellular (Fig. 5) was unexpected; it presented the conundrum of reconciling the observation that a membrane-impermeable agonist could cause accumulation of intracellular α3 AChRs (Fig. 5) with the observation that antagonists could not block up-regulation (Fig. 6), which indicated that ion flow through α3 AChRs could not be used to signal the inside of the cell to more rapidly synthesize α3 AChRs. Demonstration that the nicotine-induced increase in α3 AChRs could be prevented by antigenic modulation (Fig. 7) showed that all of the α3 AChRs affected by nicotine had been on the surface, where they could bind membrane-impermeable quaternary amine ligands. Fig. 10 depicts the mechanism of antigenic modulation. We hypothesize that chronic exposure to agonists causes α3 AChRs to assume a conformation, perhaps a desensitized conformation, that at least in SH-SY5Y cells with antigenic modulation is depicted as inducing a desensitized conformation of α3 AChRs that results in their internalization to a compartment in which as a result of a process that depends on protein synthesis, they are not immediately destroyed; ultimately they are proteolytically degraded. Antigenic modulation is depicted as a process in which cross-linking of α3 AChRs by mAbs to the extracellular surfaces of their α3 and α5 subunits causes aggregation, which speeds endocytosis and lysosomal destruction.

Fig. 10. Comparison of proposed mechanisms of agonist-induced internalization of α3 AChRs in SH-SY5Y cells with antigenic modulation of α3 AChRs. Nicotine is depicted as inducing a desensitized conformation of α3 AChRs that results in their internalization to a compartment in which as a result of a process that depends on protein synthesis, they are not immediately destroyed; ultimately they are proteolytically degraded. Antigenic modulation is depicted as a process in which cross-linking of α3 AChRs by mAbs to the extracellular surfaces of their α3 and α5 subunits causes aggregation, which speeds endocytosis and lysosomal destruction.

References


