Chronic Nicotine Exposure Differentially Affects the Function of Human α3, α4, and α7 Neuronal Nicotinic Receptor Subtypes

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ABSTRACT

Because chronic exposure to nicotine and nicotinic drugs might both activate and desensitize nicotinic acetylcholine receptors (AChRs), we sought to determine whether prolonged exposure to nicotine concentrations encountered in tobacco users differentially affects electrophysiological properties of major subtypes of human neuronal nicotinic AChRs. Xenopus laevis oocytes were injected with subunit cRNAs encoding (1) homomeric α7 AChRs, (2) heteromeric α4β2 AChRs and (3) heteromeric α3 AChRs formed from combinations of α3, β2, β4 and α5 cRNAs. Acute activation required micromolar concentrations of nicotine. Chronic exposure to submicromolar concentrations of nicotine irreversibly inactivated many α3 AChRs but inhibited α7 AChRs much less. Thus, although α3 AChRs are present in the brain in much smaller amounts than are α4β2 AChRs or α7 AChRs, α3 AChRs in brain and autonomic ganglia may be able to play a relatively large role in acute responses to endogenous ACh or subsequent doses of nicotine after chronic exposure to nicotine. The behavioral effects of nicotine may typically reflect the sustained inhibition of α4β2 AChRs and α7 AChRs in combination with the residual susceptibility of α3 AChRs and perhaps some other AChR subtypes for acute activation. Tolerance for nicotine exhibited by tobacco users may reflect the long-term irreversible functional inactivation of α4β2 AChRs and α7 AChRs produced by chronic exposure to nicotine.

Nicotine acting at neuronal nicotinic AChRs is the primary component of tobacco that drives its habitual use (Benowitz, 1996). 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, that nicotine slowly builds to a low steady concentration which causes both reversible desensitization and long-term inactivation of AChRs as well as increases in the amounts of some AChR subtypes and that smokers medicate themselves with nicotine to regulate their AChR response (Collins and Marks, 1996; Dani and Heinemann, 1996; Wonnacott et al., 1996).

Nicotine and nicotinic drugs could be important in some neurological diseases because it has been shown that a substantial decrease in nicotinic AChRs is characteristic of both Alzheimer’s and Parkinson’s disease (Lange et al., 1993; Whitehouse et al., 1988). Epidemiological studies also indicate that smoking may be protective against Parkinson’s disease and, to a lesser extent, Alzheimer’s disease (Morens et al., 1995). In Parkinson’s disease, there is loss of dopaminergic neurons due to the degeneration of the substantia nigra. It is known that presynaptic AChRs can modulate the release of dopamine (Wonnacott et al., 1996) and that nicotine can be neuroprotective against excitotoxicity (Akaike et al., 1994). These results suggest mechanisms by which nicotine might be protective against Parkinson’s disease and by which nicotinic drugs might be therapeutic. The effects of nicotine in several other diseases suggest that nicotinic AChRs may be involved in some way in their pathology or therapy. For example, nicotine from transdermal patches is effective in reducing the severity of Tourette’s syndrome (Dursun et al., 1994). As another example, it has been reported that α7 AChRs may be responsible for an attentional deficit that may be a predisposing genetic factor for schizophrenia, that α7 AChRs are reduced in brains of schizophrenia patients and that schizophrenia patients may smoke heavily to self-medicate with nicotine (Freedman et al., 1997).

Along with the synchronized activation of AChRs by a rapid bolus of nicotine, long-term activation of this agonist can lead to inactive states of these AChRs, some of which are readily reversible and others of which are not (Collins and Marks, 1996; Dani and Heinemann, 1996; Hsu et al., 1996a; Lester and Dani, 1994; Lukas, 1991). An understanding of

ABBREVIATIONS: ACh, acetylcholine; AChR, acetylcholine receptor.
the effects of chronic exposure to nicotine on various AChR subtypes might provide better insights into mechanisms of nicotine dependence, tolerance and withdrawal and into the effects of medication with nicotine or nicotinic drugs.

An AChR subtype with the subunit stoichiometry \((\alpha4)2(\beta2)\), is thought to account for most of the high affinity nicotine binding in brain (Anand et al., 1991; Flores et al., 1992; Lindstrom, 1996; Wada et al., 1989). Nearly equal amounts of a subtype thought to have an (\(\alpha7\)) subunit stoichiometry are found in brain (Alkon and Albuquerque, 1993; Anand et al., 1993; Couturier et al., 1990; Del Toro et al., 1994; Lindstrom, 1996; Schoepfer et al., 1990; Seguela et al., 1993). The \(\alpha7\) AChRs are also often found in peripheral ganglion neurons, which also express a mixture of \(\alpha3\) AChR subtypes (Conroy and Berg, 1995). The \(\alpha3\) AChRs are found in brain, although in lower amounts than \(\alpha4\beta2\) AChRs or \(\alpha7\) AChRs (Wada et al., 1989). The \(\alpha3\) forms functional AChRs in combination with \(\beta2\) or \(\beta4\) subunits (Gerzanich et al., 1995; Papke, 1992), and \(\alpha5\) subunits assemble efficiently with both combinations (Wang et al., 1996). Presumably many subtypes of AChRs are expressed in discrete populations of neurons performing particular functional roles. Many of the AChRs in brain are thought to be located presynaptically and have been implicated in facilitating release of transmitters including ACh, dopamine, glutamate and \(\gamma\)-aminobutyric acid (Collins and Marks, 1996; Gray et al., 1996; Lena and Changeux, 1997; McGhee and Role, 1995; Wonnacott et al., 1996).

Chronic exposure to nicotine has been shown to differentially affect both the amount and function of neuronal AChR subtypes. Chicken \(\alpha4\beta2\) AChRs expressed in Xenopus laevis oocytes or a permanently transfected cell line were shown to double in amount when chronically exposed to nicotine (Peng et al., 1994). The EC\(_{50}\) for upregulation was 0.2 \(\mu\text{M}\), essentially equal to the concentration of nicotine typically found in the serum of smokers (Benowitz et al., 1990). The upregulation was due to a decrease in the rate of destruction of these AChRs resulting from an inactive conformation of these AChRs (Peng et al., 1994). Chronic exposure to high concentrations of nicotine not only reversibly desensitized these AChRs but also permanently inactivated some of them (Peng et al., 1994). Similarly, human \(\alpha4\beta2\) AChRs in a permanently transfected cell line were upregulated by chronic nicotine exposure, but the amount of ACh-induced ion flux per AChR was decreased (Gopalakrishnan et al., 1996). The \(\alpha7\) AChRs and the mixture of \(\alpha3\) AChRs expressed by the human neuromastoma cell line SH-SY5Y increased by 30\% and 60\%, respectively, in response to chronic exposure to nicotine but only when extremely high concentrations were used (Peng et al., 1997). In a comparison of the electrophysiological responses to a 48-hr exposure to nicotine of rat \(\alpha4\beta2\) AChRs expressed in X. laevis oocytes, it was found that nanomolar concentrations of nicotine eliminated most \(\alpha4\beta2\) AChR function, whereas micromolar concentrations of nicotine blocked only 50\% to 60\% of rat \(\alpha3\beta2\) AChR responses (Hsu et al., 1996a).

This study compares both the short- and long-term effects of chronic nicotine treatment on electrophysiological function of cloned human \(\alpha4\beta2, \alpha3\beta2, \alpha3\beta4, \alpha3\beta3\alpha5, \alpha3\beta2\beta4\alpha5,\) and \(\alpha7\) subunit combinations expressed in X. laevis oocytes. It examines the concentration and time dependence of the responses of these AChR subtypes to acute activation by nicotine and both reversible desensitization and permanent inactivation caused by chronic exposure to nicotine.

### Methods

#### Cloning of human \(\alpha4\) subunit cDNA

The cDNA encoding the human neuronal AChR \(\alpha4\) subunit was obtained by PCR amplification of cDNA synthesized from human brain poly(A)\(^+\) RNA (Clontech, Palo Alto, CA) with StrataScript RNase H\(^-\) Reverse Transcriptase (Strategene, La Jolla, CA) using two sets of primers (forward, GCCAGCAGCCATGTGGGA; reverse, GCCATTYTATGC-ATGACTCAGT) and (forward, TGTTACGAGGCTTCTC; reverse, AGCAAGCTCCGGTTCCCTTC TAG). For subsequent cloning into vector, the product obtained with the first primer set was digested with BsmI and NsiI endonucleases. The product obtained with the second set of primers was digested with NsiI.

The 5′ end of this subunit was amplified from 5′-RACE-Ready cDNA (Clontech) using Anchor Primer supplied with the kit (CTGGTTGC-GGCCACTTCTGAAGGTTCCAGAATGCTGATG) and specific 5′ reverse primer (GCCAGGTTCCGGGACAC). The 5′-end PCR fragment was digested with ClaI and BsmI restrictions enzymes. All PCR fragments were purified by agarose gel electrophoresis using the GeneCleen II kit (BIO 101, Vista, CA). The PCR products were ligated together via BsmI and NsiI sites and cloned into the ClaI and EcoRI blunt end sites of the pBluescript II SK\(^-\) plasmid. The construct was sequenced according to the Sanger method (Sequenase Version 2.0 DNA Sequencing Kit; United States Biochemicals, Cleveland, Ohio) to verify the sequence of the human \(\alpha4\) AChR subunit (Gopalakrishnan et al., 1996).

#### In vitro transcription, oocyte isolation and cRNA injection

cDNAs encoding the human \(\alpha4\) and \(\alpha7\) subunits were cloned into a modified SP64T expression vector, \(\alpha3\) and \(\beta4\) in pcDNAI vector, and \(\alpha5\) and \(\beta2\) in pSP64A vector, using standard DNA cloning procedures (Melton et al., 1984). cRNA was synthesized in vitro using the Megascript kit (Ambion; Austin, TX).

Oocytes were obtained from X. laevis (Xenopus I, Ann Arbor, MI). The oocytes were surgically removed and placed in oocyte physiological saline containing 96 m\(\text{M}\) NaCl, 2 m\(\text{M}\) KCl, 1.8 m\(\text{M}\) CaCl\(_2\), 1 m\(\text{M}\) MgCl\(_2\), and 10 m\(\text{M}\) HEPES, pH 7.5, to which was added 50 units/ml penicillin and 50 \(\mu\text{g}\)/ml streptomycin. Oocytes were dispersed in this buffer minus Ca\(^++\), containing 2 mg/ml collagenase A (Sigma Chemical, St. Louis, MO) for 2 hr.

Stage V–VI oocytes were selected and injected with combinations of \(\alpha4\beta2, \alpha3\beta2, \beta4\alpha5,\) and \(\alpha7\) subunit cRNAs (equal weights of 5–12 ng of each subunit per AChR subtype in a total volume of 55 nl). After injections, oocytes were maintained under semisterile conditions at 18°C in Liebovitz L-15 medium (Life Technologies, Grand Island, NY) diluted by half in 10 m\(\text{M}\) HEPES buffer, pH 7.5.

#### Purification and immunoabsorption of AChRs from oocytes and solid-phase radioimmunoassays

The purification and immunoabsorption of AChRs from X. laevis oocytes as well as solid-phase radioimmunoassays were used to compare the relative amounts of AChR subunits expressed in oocytes injected with equal amounts of \(\alpha3, \beta2, \beta4\) and \(\alpha7\) subunit cRNAs. The procedures for these experiments were as previously described (Peng et al., 1994).

#### Nicotine treatment and electrophysiological recordings

Currents were measured using a standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725; Warner Instrument Corp., Hamden, CT) as previously described (Gerzanich et al., 1995). All recordings were digitized using MacLab software and hardware (AD Instruments, Castle Hill, Australia) and stored on an Apple Macintosh IIcx computer. Data were analyzed using KALEIDAGRAPH (Synergy Software, Reading, PA).

The recording chamber was continually perfused at a flow rate of 10 ml/min with the physiological saline solution containing 0.5 \(\mu\text{M}\) atropine. Application of the agonist was performed using a set of
eight glass tubes to provide rapid application as previously described (Gerzanich et al., 1995). In all cases, 100 μM ACh was used to evoke responses. ACh was used rather than nicotine to mimic physiological conditions. The concentration of 100 μM was chosen because it is higher than the EC₅₀ values of all of the three AChR subtypes yet low enough that a comparable repeat response could be obtained within 4 to 6 min after the first ACh application.

For nicotine incubations of <60 min, the oocytes remained in the recording chamber after the initial peak current recording. They were perfused with saline solution containing the appropriate concentration of nicotine. For incubations of 60 min to 48 hr, the oocytes were removed from the chamber and incubated in a separate well with 50% L-15 medium and 10 mM HEPES buffer (pH 7.5), also containing the appropriate concentration of nicotine. At various times, the oocytes were removed from the wells and returned to the chamber for responses to be measured.

**Results**

**Acute activation by nicotine.** For each AChR type, the concentration-response curves for activation by nicotine and ACh were compared, as shown in figure 1. Table 1 summarizes some of the pharmacological properties of α4β2, α3 and α7 AChRs.

α4β2 AChRs were activated by the lowest concentrations of nicotine of the three subtypes investigated. The concentration of nicotine that produced the half-maximal activation (EC₅₀) was 0.30 ± 0.04 μM. This value is particularly significant because it is close to the 0.2 μM steady-state concentration of nicotine typical of the serum of smokers (Benowitz et al., 1990). Nicotine was a full agonist compared with ACh. Injection of equal amounts of cRNA for α3, β2, β4 and α5
subunits to model autonomic neurons and characterized neur-robastoma cell lines (Conroy and Berg, 1995; Lukas et al., 1993; Peng et al., 1993; Wang et al., 1996) resulted in the expression of a mixture of α3 AChRs. These α3 AChRs had an EC50 for activation by nicotine of 3.0 ± 0.3 μM, indicating a 10-fold lower sensitivity to nicotine than that exhibited by α4β2 AChRs. The maximal current elicited by a saturating concentration of nicotine was approximately half of that elicited by ACh. Previously, we showed that nicotine is a partial agonist for human α3β2α5 AChRs but a full agonist for human α3β4α5 AChRs (Wang et al., 1996). Thus, in a mixture of these two subtypes, nicotine should behave as a partial agonist. Immunoisolation from oocytes injected with equal amounts of α3, β2, β4 and α5 AChR subunits showed that 55% of the AChRs expressed contained the β4 subunit, whereas 35% contained the β2 subunit (fig. 2). The sum of α3 AChRs containing β2 subunits with those containing β4 subunits accounts for the total, indicating that few contain both β2 and β4 subunits. As shown in detail previously (Wang et al., 1996), under those conditions all of the 3H-epibatidine-labeled AChRs immunoisolated contain α3 subunits and >60% contain α5 subunits. These results imply that with this mixture of α3, β2, β4 and α5 cRNAs in X. laevis oocytes, most of the α3 AChRs consist of either α3β2α5 or α3β4α5 AChRs but not α3β2β2α5 AChRs.

The α7 AChRs have the lowest sensitivity to nicotine of the three subtypes investigated. The α7 homomers have been shown to have an EC50 for activation by nicotine of 40 ± 2 μM (Peng et al., 1993). Nicotine was a full agonist. The responses of α7 homomers desensitize much faster than those of the other two AChR subtypes. This is a characteristic property of α7 AChRs (Couturier et al., 1990; Peng et al., 1993; Seguela, 1993).

**Long-term inhibition by nicotine.** To determine the effects of long-term exposure to nicotine, oocytes were incubated for 48 hr in various concentrations of nicotine. Then, responses from the oocytes were tested using 100 μM ACh as agonist in a perfusing solution that also contained nicotine at the concentration used for incubation. Figure 3 compares the nicotine concentration dependence of chronic inhibition with that for acute activation.

The α7 AChRs were the most sensitive of the three AChR subtypes investigated to inhibition by chronic exposure to nicotine. Complete attenuation of response was obtained for concentrations of nicotine of >0.2 μM. The IC50 value for nicotine-induced loss of response was 2.8 ± 0.41 nM.

α4β2 AChRs also decreased their response to a saturating concentration of ACh after incubation with nanomolar concentrations of nicotine. A total loss of response to ACh occurred after incubation with nicotine concentrations of >2 μM. The IC50 value for nicotine-induced loss of responsiveness was 17 ± 3.1 nM.

In contrast, for α3 AChRs responses to ACh were not completely inhibited even after incubation with 10 μM nicotine. The IC50 value for nicotine-induced loss of responsiveness in this case was 870 ± 330 nM.

**Time course of nicotine-induced inhibition.** Initial control currents were evoked with 100 μM ACh 3 days after injection of cRNA. After incubation of the oocytes in 0.2 μM nicotine, the responses to 100 μM ACh were determined initially in 0.2 μM nicotine and again after a 1-hr rinse in nicotine-free saline to permit recovery from reversible desensitization. At each time point, responses were compared with those of control oocytes that were incubated without nicotine. At all prolonged times of incubation in nicotine, ACh-evoked responses were decreased with respect to control oocytes. Results from these experiments are shown in figure 4.

The α4β2 AChRs exposed to 0.2 μM nicotine lost their response to 100 μM ACh rapidly on prolonged exposure. After 10 sec in nicotine, the response to 100 μM ACh was

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**TABLE 1**

<table>
<thead>
<tr>
<th>AChR type</th>
<th>Activation</th>
<th>Nicotine</th>
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<tbody>
<tr>
<td></td>
<td>EC50(μM)</td>
<td>IC50(μM)</td>
</tr>
<tr>
<td>α4β2</td>
<td>1.9 ± 0.31 (1.1)</td>
<td>0.017 ± 0.0031</td>
</tr>
<tr>
<td>α7</td>
<td>79.0 ± 0.5a (2.3)</td>
<td>0.0028 ± 0.00041</td>
</tr>
<tr>
<td>α3β2β4α5</td>
<td>6.8 ± 0.5 (0.8)</td>
<td>0.07 ± 0.33</td>
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<thead>
<tr>
<th></th>
<th>Inactivation</th>
<th>Equilibrium binding</th>
<th>Up-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β2</td>
<td>0.04 (1.5)</td>
<td>1.3a</td>
<td>65.0a</td>
</tr>
<tr>
<td>α7</td>
<td>0.0031 (1.3)</td>
<td>0.017 (1.0)</td>
<td>0.21b</td>
</tr>
<tr>
<td>α3β2β4α5</td>
<td>0.02 (1.5)</td>
<td>0.020b</td>
<td>100.0c</td>
</tr>
</tbody>
</table>

*From Ref. Peng et al. (1993).
*b From Ref. Peng et al. (1994) (chicken α4β2).
*c From Ref. Peng et al. (1997).
reduced only 10%, and this reduction was completely reversed within 1 hr after removal of the nicotine. However, after 100 sec in nicotine, the response decreased 50%, and even after 1 hr of rinsing, 30% of the $\alpha_4\beta_2$ AChRs appeared to be permanently inactivated. After 2.8 hr in 0.2 $\mu$M nicotine, essentially all of the $\alpha_4\beta_2$ AChRs were desensitized to a state that required 1 hr to recover from and were presumed to be permanently inactivated.

In the case of $\alpha_7$ AChRs, the response to ACh decreased by 50% within the first 100 sec of nicotine exposure. About 90% inhibition occurred within 3 hr. This occurred despite the fact that the nicotine concentration used was 35 times less than the EC$_{50}$ value for activation of $\alpha_7$ AChRs by nicotine. At any incubation time in nicotine longer than 10 sec, the extent of recovery of the response to ACh after washing was very small.

In contrast, $\alpha_3$ AChRs were relatively unaffected by prolonged exposure to 0.2 $\mu$M nicotine. After 28 hr, the response was less than the control value by only 25%. Furthermore, these AChRs exhibited almost full recovery within 1 hr, even after a 28-hr incubation in nicotine. The inactivation caused by 3 hr in 0.2 $\mu$M nicotine was determined with each of the four $\alpha_3$ AChR subtypes (fig. 5). The $\alpha_3$ AChRs containing $\beta_2$ subunits were inhibited ~50%, whereas $\alpha_3$ AChRs containing $\beta_4$ subunits were not inhibited. This indicated that the 25% inhibition observed for the $\alpha_3\beta_2\beta_4\alpha_5$ mixture (fig. 4) resulted from the $\beta_2$-containing AChRs in the mixture.

**Time course of recovery after long-term incubation in nicotine.** We also considered the time course of recovery of response after a 28-hr incubation in 0.2 $\mu$M nicotine, as shown in figure 6. At this point, 72 hr had elapsed since the oocytes were injected with cRNA, and their ability to synthesize new AChRs was probably substantially reduced due to decay of this cRNA. The limited recoveries observed after prolonged washing suggest that in these oocytes, most of the desensitization observed after 24-hr incubation reflects permanent inactivation and there was little synthesis of new AChRs.

The $\alpha_7$ AChRs exhibited little recovery of response to 100 $\mu$M ACh after a 28-hr incubation in 0.2 $\mu$M nicotine, despite 24 hr of washing.

The $\alpha_4\beta_2$ AChRs were also substantially irreversibly inhibited by a 28-hr incubation in 0.2 $\mu$M nicotine. These AChRs were inhibited to ~10% of the control response. This response recovered to as much as 40% of the control response.
within 6 hr, but then the responses began to decrease, possibly reflecting AChR turnover in the absence of new synthesis.

By contrast, α3 AChRs were inhibited by only 25% after 28 hr in 0.2 μM nicotine, and the results show a recovery rate of >95% within 1 hr after washout.

**Discussion**

We found that prolonged exposure to nicotine affects three major human neuronal nicotinic AChR subtypes differently; α4β2 AChRs and α7 AChRs were substantially inactivated by prolonged exposure to nicotine, but α3 AChR subtypes were not.

The α4β2 AChRs exhibited the highest sensitivity for acute activation by nicotine (EC50 = 300 nM) and, correspondingly, were efficiently desensitized by 48-hr incubation in low concentrations of nicotine. The much lower concentrations of nicotine required for chronic inactivation than acute activation presumably reflect the gradual accumulation of AChRs in a high affinity desensitized conformation, which can occur without necessarily gating the AChRs. After 3 hr in 0.2 μM nicotine, the response of α4β2 AChRs to a saturating concentration of ACh was virtually eliminated, and it recovered negligibly after washing for 1 hr, suggesting that irreversible desensitization had occurred. Hsu et al. (1996a) agree that chronic exposure to nM concentrations of nicotine inhibits the response of α4β2 AChRs but not α3β2 AChRs and that micromolar concentrations of nicotine eliminate the response of α4β2 AChRs. Differences in species and methods make quantitative comparisons with their IC50 values difficult. We found that prolonged exposure to nicotine affects three major human neuronal nicotinic AChR subtypes differently; α4β2 AChRs and α7 AChRs were substantially inactivated by prolonged exposure to nicotine, but α3 AChR subtypes were not.

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rating concentration of ACh recovered completely after 1 hr of washing, in contrast with the almost total loss of α4β2 AChR response.

The α7 AChRs exhibited the lowest sensitivity for acute activation by nicotine of the three subtypes (EC$_{50}$ = 40,000 nM), 13-fold lower than α3 AChRs and 133-fold lower than α4β2 AChRs. However, α7 AChRs were the most sensitive of the three subtypes to inactivation by 48-hr incubation in low concentrations of nicotine (IC$_{50}$ = 2.8 nM), probably reflecting the rapid desensitization characteristic of α7 AChRs (Aldkon and Albuquerque, 1993; Couturier et al., 1990; Gerzanich et al., 1994, 1995; Lindstrom, 1996; Peng et al., 1993; Seguela et al., 1993). The α7 AChRs were 6-fold more sensitive than α4β2 AChRs and 310-fold more sensitive than α3 AChRs.

EC$_{50}$ values for activation by ACh and nicotine of cloned human α4β2 AChRs and α7 AChRs determined here using a set of glass tubes to ensure rapid application agree well with values obtained for these cloned AChRs by others using a millisecond-resolution multibarrel puff technique on transfected cells (Buisson et al., 1996; Gopalakrishnan et al., 1995).

Irreversible inactivation may be related to AChR up-regulation, which is another phenomenon associated with chronic exposure of nicotinic AChRs to nicotine. Previously, we showed that the upregulation of α4β2 AChRs occurs due to a decrease in AChR turnover that could be associated with a change in AChR conformation not associated with channel opening (Peng et al., 1994). It is not clear whether this conformation is identical to a reversibly desensitized or an irreversibly inactivated conformation. It is clear that despite an increase in the number of AChRs as a result of chronic exposure to nicotine, there is a net decrease in function of α4β2 AChRs and α7 AChRs. Recent findings also show that sustained exposure of α4β2 AChRs to nicotine increases the phosphorylation of the α4 subunit (Hau et al., 1996b; Molinari et al., 1996). Further investigations of this mechanism may lead to a clearer picture of the relationship between the AChR conformations and post-translational modifications associated with up-regulation and those associated with functional inactivation.

The tolerance to increased nicotine doses that is characteristic of tobacco users (Benowitz, 1996; Collins and Marks, 1996; Dani and Heinemann, 1996) can be explained by the net decreases in α4β2 AChR and α7 AChR function that we have observed after chronic exposure to nicotine. These decreases in function occur despite an increase in the amount, especially of α4β2 AChRs, induced by chronic exposure to nicotine (Peng et al., 1994, 1997). Permanent inactivation of α4 or β7 AChRs coupled with a slow rate of resynthesis may account for the weeks of benefit reported for Tourette’s syndrome patients treated for only 2 days with transdermal nicotine patches (Dursun et al., 1994). A particularly good example of nicotine acting in vivo as a time-averaged antagonist is the effect of nicotine on prolactin release in the rat (Hulihan-Giblin et al., 1990a, 1990b; Sharp and Beyer, 1986). A single intravenous injection of nicotine causes an increase in serum prolactin concentration as a result of activating AChRs in the hypothalamus. This response desensitizes rapidly and for a long duration, resulting in little or no response 1 or 6 hr later. Chronic treatment with nicotine by injections twice a day for 10 days prevented any acute response to nicotine despite provoking an increase in hypothalamic [3H]ACh binding sites. After this chronic nicotine treatment, 8 to 14 days were required for the response to acute nicotine treatment and the amount of AChR to return to normal, presumably as a result of turnover of permanently inactivated AChRs.

Gene knockout experiments also suggest that it is reasonable to think that chronic exposure to nicotine in tobacco users could be associated with the net loss of α4β2 AChR and α7 AChR function but not great neurological impairment. Knockout of the β2 subunit gene in mice eliminates the high affinity binding of nicotine in the brain that would be expected from the loss of α4β2 AChRs (Picciotto et al., 1995). Knockout of the α7 subunit gene in mice eliminates the high-affinity binding of α-bungarotoxin in brain that would be expected from α7 AChRs (Orr-Urtreger et al., 1996). In neither case do these knockout mice exhibit gross behavioral or anatomic anomalies.

In a chronic smoker with a typical serum concentration of nicotine of 0.2 μM (Benowitz et al., 1990), virtually all α4β2 AChRs would be inactivated, as would 90% of their α7 AChRs, but only 20% of their α3 AChRs would be inactivated, and only these could quickly recover as the serum nicotine concentration dropped after a few hours without smoking. Thus, the behavioral effects and reward of smoking are likely to depend on the inactivation of α4α2 AChRs and α7 AChRs while leaving α3 AChRs available to respond to the micromolar boluses of nicotine available quickly after inhalation of smoke (Benowitz, 1996). The first cigarette of the morning is generally regarded as the most rewarding (Benowitz, 1996), which might result either from increased sensitivity or resynthesis of α4β2 AChRs and α7 AChRs after overnight abstinence or as a relief from withdrawal symptoms as these two AChR subtypes are quickly returned to their chronically inactivated states. Neuronal synaptic mechanisms can be complex. In ciliary ganglia, postsynaptic α3 AChRs combine with perisynaptic α7 AChRs to contribute to a high safety factor for neurotransmission, and neurotransmission can occur even if the α7 AChRs are blocked with α-bungarotoxin (Zhang et al., 1996). At this type of synapse, the α7 AChR component of the postsynaptic current would be blocked by the chronic presence of nicotine, but transmission could still occur through α3 AChRs.

The α4 (Wada et al., 1989) and α7 AChRs (Cimino et al., 1992; Del Toro et al., 1994; Seguela et al., 1993) are expressed in many areas of the brain, whereas α3 AChRs can be found mostly in the peripheral nervous system (McGehee and Role, 1995; Orr-Urtreger et al., 1996; Wada et al., 1989; Whiting et al., 1991). In the brain, α3 AChRs have been thought to be localized to areas known to be directly involved in the reward mechanisms of drug abuse, including the locus ceruleus, ventral tegmental area and substantia nigra (Wada et al., 1989). Recent evidence, however, suggests that many of the dopamine-containing regions of the brain associated with addiction or Parkinson’s disease may in fact contain α6 rather than α3 (LeNovère et al., 1996). The α6 subunits are closely related to α3 subunits in sequence. Their ability to function as parts of AChRs has only recently been demonstrated (Gerzanich et al., 1997). The α6β4 AChRs were found to differ pharmacologically from α3β4 AChRs, with nicotine behaving as an 18% partial agonist with a prolonged inhibitory effect. Therefore, investigations of the acute
and chronic effects of nicotine on α7 AChRs will be important in the future. It remains to be determined which AChR subtypes are associated with particular components of the behavioral responses to chronic exposure to nicotine and how these AChR subtypes act by presynaptic and postsynaptic mechanisms in various circuits to produce these behavioral effects.

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