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Long-Term Exposure to Nicotine Modulates the Level and Activity of Acetylcholine Receptors in White Blood Cells of Smokers and Model Mice

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ABSTRACT

Long-term consumption of tobacco by smokers causes addiction and increases the level of neuronal nicotinic acetylcholine receptors (nAChRs) in the brain, a phenomenon known as up-regulation. Here, we show that up-regulation of specific nAChR subunits takes place in white blood cells (WBCs) of smokers and mice subjected to long-term administration of nicotine. The basal level of α-bungarotoxin binding site, which corresponds to the homomeric α7 nAChR subtype, was not affected in WBCs of both smokers and mice administered nicotine. In contrast, epibatidine (EB) binding sites, which correspond to heteromeric nAChR subtypes, were detected in WBCs of smokers but not in WBCs of nonsmokers. The number of EB binding sites significantly decreased after incubation of the smokers' WBCs for 3 days in nicotine-free culture medium. In WBCs of wild-type mice, basal level of EB binding sites was detected before nicotine administration. This basal level is reduced by ~60% in knockout mice lacking the genes encoding either the β2 or the α4 receptor subunits. Additional analysis of knockout mice revealed that the remaining ~40% do not undergo up-regulation, indicating that the α4/β2 subunits comprise the up-regulated nAChRs. We further found that up-regulation in mouse WBCs is accompanied by a significant decrease in the capacity of the up-regulated receptor channels to convey calcium ions. The phenomenon of nAChR up-regulation in WBCs provides a simple tool to evaluate and study tobacco addiction.

Tobacco consumption is a highly addictive behavior that is estimated to be the major cause of preventable death in developed countries (Peto et al., 1992). Nicotine, an alkaloid found in high concentrations in tobacco leaves, induces and maintains this addictive behavior by interacting with neuronal nicotinic acetylcholine receptors (nAChRs). These receptors are known to modulate the activity of the central nervous system's reward centers (Picciotto et al., 1998; Dani et al., 2001; Buisson and Bertrand, 2002; Mansvelder and McGehee, 2002; Shoaib et al., 2002). Over time, long-term activation of nAChRs alters the properties of the neuronal circuits, which leads to complex behavior such as dependence, tolerance, sensitization, and craving (Koob et al., 1998). The involvement of these receptors in physiological and pathophysiological processes (Lindstrom, 1997) relies, in part, on molecular mechanisms such as activation, desensitization, and up-regulation of different nAChR subtypes after long-term exposure to nicotine. Receptor up-regulation, which is characterized by an increased number of high-affinity nicotine binding sites, can be measured postmortem in the brains of smokers (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999; Paterson and Nordberg, 2000) and rodents administered nicotine on a long-term basis (Flores et al., 1992; Sanderson et al., 1993). Up-regulation of nAChRs also occurs in vitro in cell lines continually exposed to nicotine (Peng et al., 1994; Buisson and Bertrand, 2001). Yet a few crucial questions regarding the relationship between tobacco consumption behavior and up-regulation remain unanswered. For instance, is nicotine the only molecule among the 4000 substances found in tobacco that causes and maintains up-regulation in the smoker's brain? What are the nAChR subtypes involved in this phenomenon, and are the up-regulated receptors functional?

Because changes in the expression of neuronal nAChRs are

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; EB, epibatidine; α-BGT, α-bungarotoxin; WBC, white blood cell; WT, wild type; DHβE, dihydro-β-erythroidine; PMN, polymorphonuclear.

difficult to investigate in human brains during smoking and smoking cessation, the study of up-regulation in tissues, which can be readily sampled, might shed light on these questions. In addition to its effects on the central nervous system, nicotine is known to affect many other tissues such as the respiratory tract, skin, and vascular and immune tissues (Grando et al., 1995; Macklin et al., 1998; Maus et al., 1998; Sato et al., 1999). Furthermore, nAChRs have already been found in lymphocytes (Skok et al., 2003) as well as in polymorphonuclear (PMN) leukocytes (Benhammou et al., 2000). In this context, we sought to investigate the regulation of nAChRs in blood cells of smokers and of wild-type (WT) and nAChR knockout mice continually administered nicotine.

Materials and Methods

Clinical Data. Volunteer smokers were recruited from the Smoking Cessation Center at Albert Chenevier Hospital (Créteil, France). The intent and details of the proposed research were thoroughly explained to the smokers and nonsmokers, and written consent was obtained from each individual participating in this study. Our experimental protocol was approved by the regional ethical committee (Comité Consultatif pour la Protection des Personnes et la Recherché Biomédicale). Only smokers with a score ≥2 in the Fagerström Test for Nicotine Dependence were included (Heatherton et al., 1991). Subjects with a history of alcoholism or psychiatric illness other than depression were excluded from the study. The individuals examined had no history of arrhythmias or recent myocardial infarction, were not pregnant or breastfeeding, and had no history of glaucoma, urinary retention, thyroid disease, epilepsy, or chronic convulsive syndromes. The sample consisted of 92 smokers: 47 women (mean age, 45.5 ± 8.3 years) and 45 men (mean age, 48.5 ± 9.1 years). The nonsmoking group was composed of 26 women (mean age, 46.1 ± 6.5 years) and 24 men (mean age, 44.2 ± 6.5 years). Blood was taken 2 to 3 h after the last cigarette. Levels of cotinine and creatinine were measured in urine samples and expressed as a cotinine/creatinine ratio to adjust for variable rates of urine secretion.

Isolation of PMN Leukocytes from Human Blood. Human PMN leukocytes were isolated according to the method described by Cabanis et al. (1994), with slight modifications. In brief, 20 ml of fresh heparinized blood was diluted with an equal amount of 0.1 M phosphate-buffered saline, pH 7.4, and transferred into 10-ml tubes (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). After centrifugation at 400*g* for 30 min, the pellet was resuspended in 40-ml isotonic ammonium chloride solution at 4°C. Twenty minutes later, the cell suspension was centrifuged at 160*g* for 10 min, and the white pellet was washed twice in 10 ml of Hanks' buffer. Cell viability was determined by Trypan blue exclusion. Protein determination was performed using the Lowry method (Lowry et al., 1951).

Radioligand Binding Assay. Binding assays were performed on intact purified human PMN leukocytes and mouse spleen cells. Human PMN leukocytes (5×10^6) or mouse splenocyte cells were incubated at 25°C with 10 nM of either [^{3}H]EB or ^{125}I-α-BGT for 30 or 60 min, respectively. The radiolabeled ligands were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Specific binding was defined as the difference between total binding and binding in the presence of 1 μM α-BGT or 100 μM nicotine, respectively. For saturation studies, increasing concentrations of [^{3}H]EB (1 pM to 10 nM) or ^{125}I-α-BGT (1 pM to 10 nM) were used. Specific binding was defined as described above for each ligand concentration. Bound and free ligands were separated by rapid vacuum filtration through Whatman GF/B fiberglass filters (PolyLabo) presoaked with ice-cold rinse buffer (5 mM KH_2PO_4, 20 mM Na_2HPO_4, and 100 mM NaCl, pH 7.4) plus 0.1% milk. Filters were quickly rinsed three times with 5 ml of ice-cold buffer and transferred to scintillation vials containing 4 ml of Picofluor 30 (PerkinElmer Life and Analytical Sciences, Boston, MA). The filters were counted with a scintillation β-counter (for [^{3}H]EB) or a γ-counter (for ^{125}I-α-BGT).

Ex Vivo Studies. Freshly purified human PMN leukocytes were incubated with or without nicotine for 3 days at 4°C. At the end of incubation, cells were washed twice in Hanks' buffer to eliminate the presence of nicotine, and then binding experiments were performed as described above.

Animals. We used age-matched (2–4 months old) male wild-type and mutant mice with common C57BL/J6 background. Four different knockout mice were used, lacking either β2 (Picciotto et al., 1998), α4 (Marubio et al., 1999), α7 (Orr-Urtreger et al., 1997), or α4β2 nAChR subunits. Mice were housed in a quiet, temperature-controlled room (22–23°C) under a 12-h light-day cycle and were provided with water and dry food pellets ad libitum. All procedures conformed to the guidelines of the Centre National de la Recherche Scientifique.

Preparation of Mice Spleen Cells. White cells were collected from sacrificed mice. In brief, spleen was carefully removed, placed in Hanks' balanced salt solution, pH 7.4, at room temperature, and pressed through stainless steel mesh. Splenocyte suspension was washed once in Hanks' saline buffer and centrifuged at 200*g* for 5 min. Red blood cells were lysed by isotonic ammonium chloride solution, and splenocytes were resuspended in Hanks' saline buffer. Cell viability exceeded 95% by Trypan blue exclusion.

In Vivo Prolonged Treatment. Wild-type mice were divided into two groups, receiving either (−)-nicotine at calculated concentration of 2.4 mg/kg/day or saline (control group), using mini-osmotic pumps (model 2004; Alzet, Cupertino, CA). The latter were transplanted subcutaneously after the mice were anesthetized with 5 mg/kg xylazine and 25 mg/kg ketamine. Twenty-eight days after transplantation, mice were killed, the spleens were carefully removed, and splenocytes were prepared as described above.

Measurements of Intracellular Calcium Concentration. Following the methods of Stauderman et al. (1998), splenocytes were loaded with 5 μg/ml Indo-1AM (Molecular Probes, Eugene, OR) for 45 min at 37°C in the dark. Flow-cytometry analysis was performed using the BD LSR system (BD Biosciences, San Jose, CA) to determine the ratio of bound to free dye in splenocytes as a function of the amount of free Ca^{2+} in the cytosol, expressed as the ratio of λ^{424} (high calcium) to λ^{475} (zero calcium). Cells were maintained at 37°C during analysis. Debris and cell aggregates were initially excluded from analysis. Dead cells were removed from the analysis by gating out nonfluorescent cells, as described by Lecoeur et al. (1997). Instrument settings were adjusted to establish a ratio baseline response for unstimulated cells. Nicotine was introduced after approximately 45 s. The specificity of the response to nAChRs was determined by preincubating the cells with the nAChR antagonist dihydro-β-erythroidine (DHβE) for 10 min before calcium measurement.

Statistical Analysis. The binding parameters K_d and B_{max} were determined from Scatchard plots using a nonlinear curve-fitting program with the Micropharm software (Urien, 1995). All results were expressed as mean ± S.D., and data were analyzed using Student's *t* test.

Results

Up-Regulation of EB Binding Sites in Blood Cells of Smokers. To determine whether smoking habits alter the levels of nAChRs in biological samples readily accessible to the clinician, polymorphonuclear neutrophils from the blood of smokers ($n = 92$) and nonsmokers ($n = 50$) were analyzed under nearly saturating concentrations with the radiolabeled ligands EB and α-BGT. EB binds to neuronal heteromeric nAChRs and α-BGT to homomeric α7 nAChRs. As shown in

Fig. 1A, smokers had on average $4.6 \pm 0.3 \times 10^3$ [^{3}H]EB binding sites per PMN leukocyte, whereas no such sites could be detected in nonsmoker cells. Note that no EB binding sites were detected in PMN leukocytes of 18 smokers (excluded from the mean calculation). In contrast, similar amounts of ^{125}I-α-BGT binding sites were detected in smokers and nonsmokers, with $44.8 \pm 7.5 \times 10^3$ and $36.5 \pm 5.9 \times 10^3$ binding sites per leukocyte, respectively (Fig. 1B).

EB binding sites of PMN leukocytes sampled from the blood of smokers ($n = 3$) were further analyzed by Scatchard analysis, which revealed the existence of two classes of EB binding sites. These classes display high and low dissociation constants (K_d) of 56.3 ± 27.8 pM and 2.1 ± 0.4 nM, respectively (Fig. 1C). The maximum number of [^{3}H]EB binding sites (B_{max}) was $4.9 \pm 2.0 \times 10^3$ per cell, of which 14% represent the high-affinity class. As for α-BGT binding, a single class of high-affinity binding sites was found, which displayed a K_d value of 2.8 ± 1.5 nM and a B_{max} of $54.8 \pm 4.0 \times 10^3$ binding sites per PMN leukocyte (Fig. 1D).

We then sought to determine whether nicotine alone triggers the up-regulation of [^{3}H]EB binding sites observed in smokers' leukocytes. PMN leukocytes were cultured for 3 days in the absence or presence of nicotine. Without nicotine in the culture medium, nonsmoker leukocytes were still devoid of [^{3}H]EB binding sites, whereas the number of sites measured in smoker leukocytes were decreased by almost two thirds compared with cells analyzed immediately after blood sampling (Fig. 1, A and E). In the case of leukocytes of nonsmokers, upon the addition of 1 μM, 50 μM or 1 mM nicotine to the culture medium, the number of [^{3}H]EB binding sites per cell sharply increased from 0 to $2.7 \pm 0.6 \times 10^3$, $4.9 \pm 0.3 \times 10^3$, and $5.1 \pm 0.4 \times 10^3$, respectively. In leukocytes of smokers, under the same nicotine concentrations, the number of [^{3}H]EB binding sites per cell increased from $1.5 \pm 0.2 \times 10^3$ to $2.8 \pm 0.5 \times 10^3$, $5.3 \pm 0.8 \times 10^3$, and $4.3 \pm 0.5 \times 10^3$, respectively (Fig. 1E).

The relationship between EB binding site levels and tobacco addiction of donor samples was evaluated by using the Fagerström Test for Nicotine Dependence (Heatherton et al., 1991) and daily cigarette consumption (number of cigarettes per day) as indicators of addiction. We found a positive (yet moderate) correlation between the number of [^{3}H]EB binding sites in PMN leukocytes of smokers and the Fagerström index ($r = 0.520$, $P < 0.0001$) (Fig. 1F) and daily cigarette consumption ($r = 0.533$, $P < 0.0001$) (Fig. 1G).

An Animal Model for nAChR Up-Regulation in WBCs. Given the availability of various nAChR knockout mice and the fact that large amounts of white blood cells can be readily collected from the mouse spleen, we chose this animal as a model to further investigate the regulation of

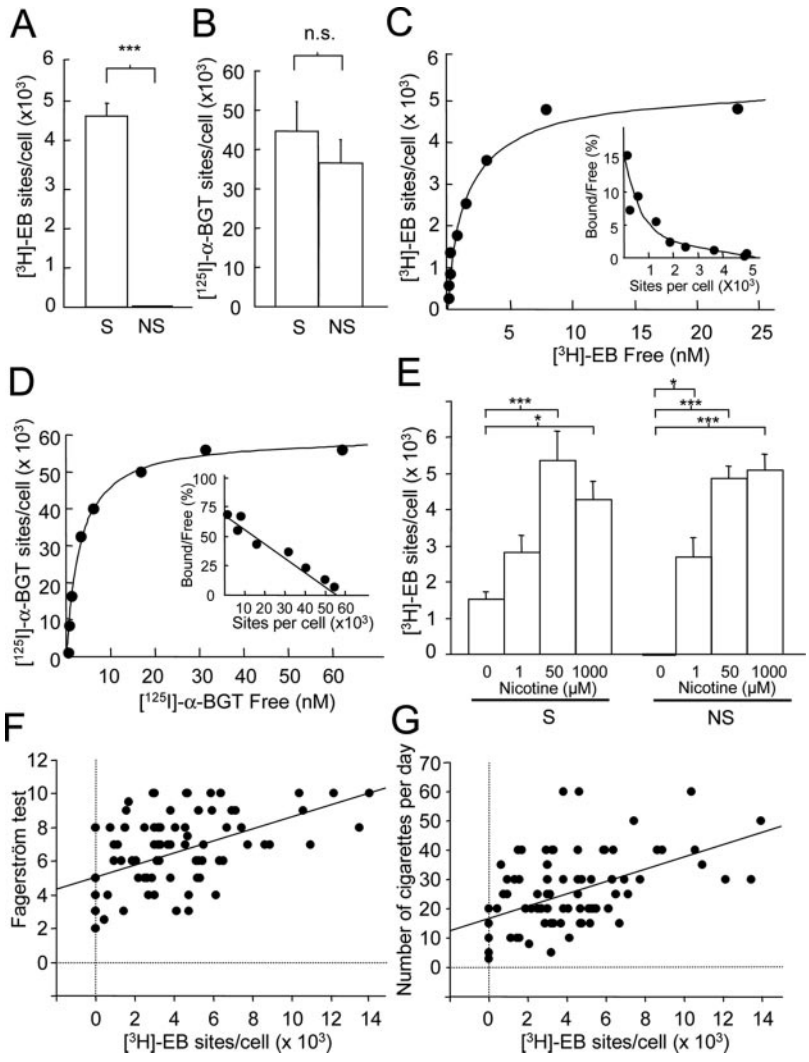

Fig. 1. EB binding sites are exclusively detected in leukocytes of smokers. A and B, levels of nicotinic binding sites in human PMN leukocytes. For [^{3}H]EB binding, 74 blood samples of smokers (S) and 50 of nonsmokers (NS) were examined. For ^{125}I-α-BGT binding, blood samples of 92 smokers (S) and of 50 nonsmokers (NS) were tested. C and D, representative isotherms of [^{3}H]EB and ^{125}I-α-BGT binding to PMN leukocytes of a smoker. Insets, Scatchard analyses of the binding isotherms. E, levels of EB binding sites in PMN leukocytes isolated from the blood of smokers (S) or nonsmokers (NS) as measured after 3 days of incubation in the absence ($n = 6$) or presence of 1 μM ($n = 3$), 50 μM ($n = 3$), and 1 mM ($n = 6$) nicotine. F and G, correlation between the number of EB binding sites ($n = 92$) measured in PMN leukocytes of smokers with the Fagerström Test for Nicotine Dependence (F) and with the number of cigarettes smoked per day (G). Correlation values are moderate: $r = 0.520$, $P < 0.0001$ (F) and $r = 0.533$, $P < 0.0001$ (G). ★★★, $P < 0.001$; ★, $P < 0.05$; n.s., not significant.

nAChRs in leukocytes. The identification of nAChRs expressed by mouse blood cells was first determined by measuring [^{3}H]EB binding sites and ^{125}I-α-BGT binding sites in wild-type mice splenocytes (Fig. 2, A and B). Both EB and α-BGT binding experiments revealed a single class of sites, with K_d values of 3.6 $\pm$ 0.7 and 1.5 $\pm$ 0.9 nM, respectively. The B_{max} values for EB and α-BGT were 4.4 $\pm$ 0.6 $\times$ 10^3 and 32.0 $\pm$ 2.0 $\times$ 10^3 binding sites per cell, respectively.

To then study in vivo the long-term effect of nicotine on the expression of nAChR subtypes in splenocytes and in the brain, mice were exposed continually to nicotine (2.4 mg/kg/day) or saline over a period of 28 days. Continuous nicotine delivery was achieved with mini-osmotic pumps transplanted subcutaneously. This dose yields stable levels of nicotine in the plasma (Murrin et al., 1987) (30–35 ng/ml), similar to those reported for smokers consuming 30 cigarettes per day (Huston-Lyons and Kornetsky, 1992) (40–42 ng/ml). After nicotine administration, a significant increase of EB binding sites was measured in both mice splenocytes (1.9 $\pm$ 0.2-fold) and mice brain (1.48 $\pm$ 0.1-fold) (Fig. 2, C and D). In contrast, as in smoker leukocytes, the administration of nicotine did not affect the level of α-BGT binding sites in mouse brain or splenocytes (Fig. 2, E and F).

The identification of nAChR subtypes expressed in splenocytes was then explored by analyzing binding on splenocytes prepared from different nAChR knockout mice. As shown in Fig. 3A, specific [^{3}H]EB binding sites ($\times$ 10^3) per cell amounted to 4.6 $\pm$ 0.4 in control wild-type mice, 1.5 $\pm$ 0.1 in knockout mice for the α4 subunit ($\alpha4^{-/-}$), 1.9 $\pm$ 0.2 in $\beta2^{-/-}$, and 1.9 $\pm$ 0.1 in $\alpha4^{-/-}\beta2^{-/-}$ mice. These results indicate that the largest population of EB binding sites in splenocytes is the $\alpha4\beta2$ nAChR subtype. The lack of α-BGT binding sites in $\alpha7^{-/-}$ mice indicates that α-BGT binding in splenocytes is exclusively contributed by the α7 nAChR (Fig. 3B). Long-term exposure to nicotine did not affect the level of EB binding sites in brain or splenocytes of $\beta2^{-/-}$ mice (Fig. 3, C and D).

Up-Regulation of nAChRs Is Accompanied by a Decrease in Calcium Permeability. Because most nAChR channels are capable of conducting calcium, we evaluated Ca^{2+} permeability in splenocytes expressing up-regulated nAChRs. To calibrate the system, we first measured nicotine-elicited increase of intracellular Ca^{2+} in splenocytes sampled from mice that were not preadministered nicotine (Fig. 4A). As a reference for the maximal increase of cytosolic calcium in living cells, the increase in fluorescence ratio (equivalent to calcium uptake) was monitored after the addition of ionomycin, a calcium ionophore which forms Ca^{2+}-permeable pores in the cell membrane. The rapid increase in nicotine-elicited calcium uptake (Fig. 4A) enabled us to plot a dose-response curve and to determine an EC_{50} value of 13 $\pm$ 6 μM (n = 4) (Fig. 4B). To ascertain that Ca^{2+} ions enter the cells through nAChR channels, the effect of a competitive nAChR antagonist (DHβE) was determined. Indeed, Fig. 4C shows that DHβE completely blocks the basal Ca^{2+} uptake. The maximal nicotine-elicited response measured in splenocytes of $\beta2^{-/-}$ mice was two times lower than that observed in WT mice (Fig. 4D). Measurements performed in splenocytes sampled from WT mice that were preadministered with nicotine showed unexpectedly low calcium uptake, comparable with those observed with $\beta2^{-/-}$ splenocytes (Fig. 4D).

Discussion

Addiction to Nicotine Correlates with Up-Regulation of Acetylcholine Receptors in Human Polymorphonuclear Leukocytes. Tobacco smoking is considered to cause an increase of nicotine- and EB-binding sites in the

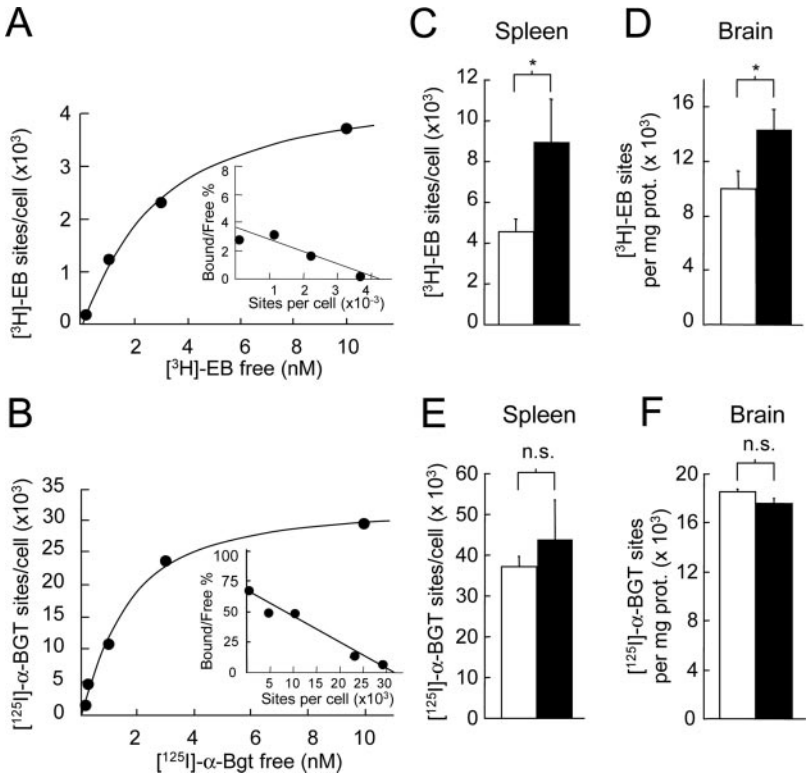

Fig. 2. Up-regulation of nAChRs in splenocytes and brain tissue of mice exposed continually to nicotine. A and B, ligand-binding isotherms and Scatchard plots (insets) of [^{3}H]EB and ^{125}I-α-BGT binding to mice splenocytes (n = 3). C and D, levels of EB binding sites in splenocytes and brain tissue of WT mice that were continually administered saline (□) or nicotine (■) for 28 days (n = 6 per group). E and F, level of ^{125}I-α-BGT binding sites in spleen and brain of mice (n = 6 per group) treated as in C and D. ⋆, $P < 0.05$; n.s., not significant.

central nervous system, as observed in postmortem brains of smokers (Benwell et al., 1988). The presence of nAChR in blood cells (Benhammou et al., 2000) makes it possible to study nAChRs in PMN leukocytes from samples of living individuals. However, thus far, this characterization has been confined to analysis of [^{3}H]nicotine binding sites (Benhammou et al., 2000). Here we discovered that unlike the case of α-BGT binding sites, which are found in PMN leukocytes of both smokers and nonsmokers, EB binding sites are only detectable in PMN leukocytes of smokers (Fig. 1, A and B). We do not exclude the existence of a very low level of EB binding sites in cells of nonsmokers. It is possible that such a low level is lower than the threshold of our detection system. In any case, our findings strongly indicate that EB binding sites, which correspond to neuronal heteromeric nAChRs, undergo significant up-regulation as a consequence of tobacco smoking. Homomeric α7 nAChRs, which did not undergo up-regulation in smokers' leukocytes tested here, were previously shown to be up-regulated in vitro in SH-SY5Y cells after exposure to nicotine concentrations greater than 10 μM (Peng et al., 1997). However, the concentration of nicotine in smokers' blood increases to a maximum of 1 μM during cigarette smoking (Benowitz and Jacob, 1990), which is well below the up-regulation threshold of α-BGT binding sites found by Peng et al. (1997).

Although tobacco contains high amounts of nicotine, it also contains more than 4000 other molecules, many of which have neuroactive properties (Fowler et al., 1996). An inevitable question therefore arises: Is nicotine sufficient to trigger and maintain up-regulation in PMN leukocytes? To address this question, we studied this phenomenon ex vivo in cells isolated from human blood. We found that incubation of PMN leukocytes of nonsmokers with nicotine for a few days triggers an increase in EB binding sites, from nothing to levels similar to those monitored in cells of smokers immediately after sampling (compare Fig. 1, A and E). It is interesting that the level of EB binding sites in cells isolated from the blood of smokers decreased by 3-fold after 3 days in nicotine-free medium. This correlates with the observation that the increased level of nicotine-binding sites in smokers' blood persists for at least a few days after smoking cessation (Lebargy et al., 1996). Taken together, these results indicate that prolonged exposure of tobacco smokers to nicotine cause specific up-regulation of EB binding sites in PMN leukocytes.

Molecular Study of nAChRs Involved in Up-Regulation in Mice. The pharmacological experiments performed on wild-type and knockout mice lacking the α7, α4, β2, or α4 plus β2 subunits made it possible to identify the nAChR subtypes expressed in splenocytes. Three types of receptors were thus identified in mouse splenocytes: 1) α7 receptors, which bind α-BGT and disappear in $\alpha7^{-/-}$ mice, indicating that α-BGT bindings sites on these cells are contributed solely by α7 nAChR (Fig. 3B); 2) $\alpha4\beta2$ heteropentamers, which contribute to most of the high-affinity EB binding seen in wild-type mice and which is drastically reduced in $\alpha4^{-/-}$, $\beta2^{-/-}$, or $\alpha4^{-/-}\beta2^{-/-}$ mice (Fig. 3A); and 3) heteropentamers which bind EB and which remain in $\alpha4^{-/-}\beta2^{-/-}$ mice (Fig. 3A). The level of the latter, which probably corresponds to the $\alpha3\beta4$ subtype, does not vary in the splenocytes and brain of $\beta2^{-/-}$ mice administered with nicotine (Fig. 3, C and D). It can thus be concluded that the α4 and β2 subunits comprise the up-regulated nAChRs (Fig. 3, C and D). Furthermore, it can be safely assumed that $\alpha4\beta2$ receptors also correspond to the receptor subtypes that are up-regulated in the PMN leukocytes and brain of smokers as well. Up-regulation of nicotine-binding sites was reported not to be associated with

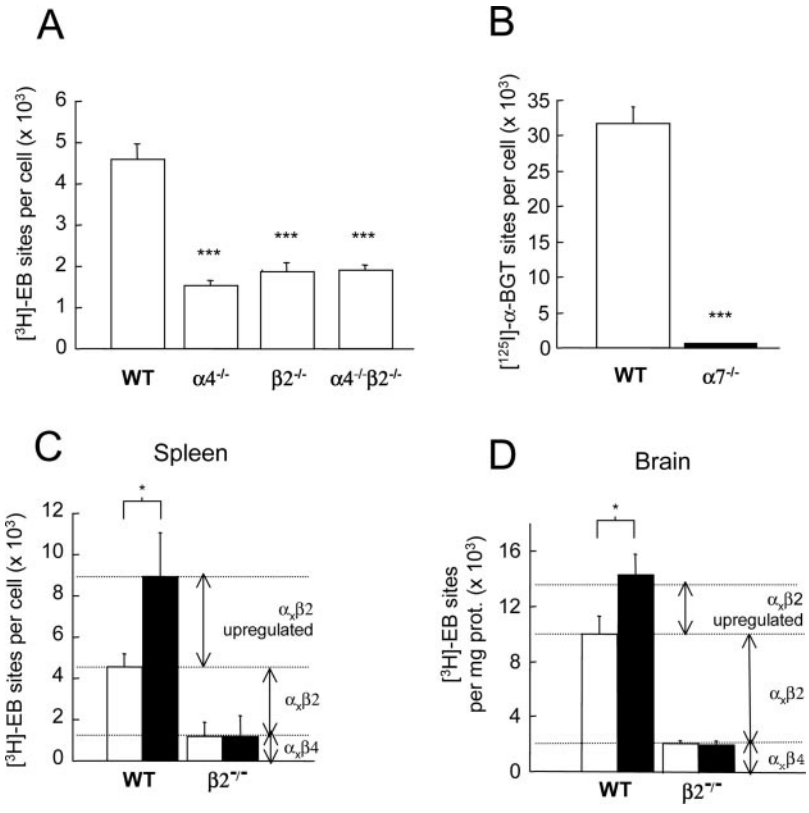

Fig. 3. Subunits implicated in up-regulation of nAChRs in splenocytes and brain tissue. A, levels of [^{3}H]EB binding sites in WT and knockout mice ($-/-$) lacking either α4, β2, or both α4 and β2 subunits ($n = 3$ per group). B, levels of ^{125}I-α-BGT binding sites in WT and mice lacking the α7 subunit ($\alpha7^{-/-}$). C and D, levels of EB binding sites in the spleen and brain of WT and $\beta2^{-/-}$ mice continually administered saline (□) or nicotine (■). α_x may correspond to α3, α4, or α6 nAChR subunits, which are most likely to form functional heteropentamers with β2 or β4. ⋆, $P < 0.05$; ⋆⋆⋆, $P < 0.001$.

changes in levels of brain $\alpha 4$ or $\beta 2$ mRNA (Pauly et al., 1996). Although the molecular underpinning of this phenomenon remains to be elucidated, several mechanisms other than transcriptional processes have been proposed. These were post-translational recruitment of pre-existing subunits (Wang et al., 1998), stabilization of assembled but unstable pentamers, and/or protection against turnover (Peng et al., 1994).

In contrast to the muscle-type nAChR, neuronal nAChRs are highly permeable to calcium (Albuquerque et al., 1996). Taking advantage of this property, we assessed the channel activity of nAChR in mice splenocytes after administration of nicotine. Despite the increase in the number of EB binding sites, there was a significant decrease in nicotine-evoked calcium uptake in splenocytes sampled from WT mice that were pre-exposed to nicotine (Fig. 4D). Our observation might imply that prolonged nicotine exposure in mice induces several phases of nAChR functional loss. Such a loss might be an outcome of desensitization and/or persistent deactivation (Mansvelder et al., 2002). Up-regulation of human $\alpha 4\beta 2$ nAChR, expressed in *Xenopus laevis* oocytes, was found to correlate with receptor desensitization, which prevents any ionic flow (Fenster et al., 1999). In contrast, human $\alpha 4\beta 2$ nAChRs expressed in human embryonic kidney 293 cells could be activated even after a long pre-exposure to nicotine, displaying higher apparent affinity of activation, higher current amplitudes, and less desensitization (Buisson and Bertrand, 2001). However, as discussed by Buisson and Bertrand (2001, 2002), the effects of long-term pre-exposure to nicotine might depend on the type of cell-expressing system, the conditions of cell maintenance, and the endogenous activity of intracellular factors such as kinases or phosphatases that could be different from one cell type to another. Moreover, sodium influxes, which were measured by Buisson and Bertrand (2001), do not necessarily indicate permeability of the up-regulated receptors to calcium, as is the case of the muscle-type nAChR. Further work is warranted to uncover how the balance between up-regulation and persistent decrease in calcium uptake affects nicotine dependence and immune responses. Although the role of nAChRs in the modulation of immune responses was beyond the scope of this study, it is important to note that many of the adverse effects of smoking might result from the ability of nicotine to decrease the immune response (Sopori, 2002). For instance, activation of the homomeric $\alpha 7$ AChR by acetylcholine released from the vagus nerve endings in the thymus can specifically inhibit macrophage activation (Wang et al., 2003). The relationships between smoking-induced up-regulation of nAChRs, nicotine-dependent loss of calcium transport via nAChRs, and alterations in the immune response are currently under investigation.

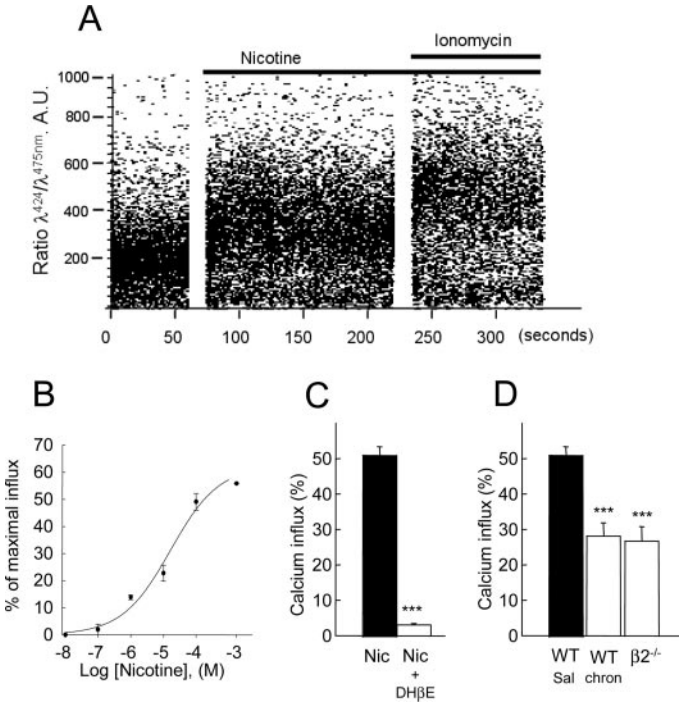

Fig. 4. Nicotine-elicited calcium uptake decrease in splenocytes of mice continually administered nicotine. A, a calibrating experiment of Ca^{2+} uptake measured in splenocytes of WT mice that were not pre-exposed to nicotine. Application bars correspond to stimulation with nicotine (10^{-4} M) and ionomycin (10 μM). B, dose-response curve corresponding to the percentage of the maximal normalized response plotted as a function of nicotine concentrations. The maximal calcium uptake is obtained in the presence of 10 μM ionomycin in WT mice. Values are mean $\pm$ S.D. (n = 4 mice). C, normalized response induced in splenocytes of WT mice by nicotine and blocked by 1 mM DHβE, a specific competitive antagonist of nAChRs. D, Ca^{2+} uptake in response to 0.1 mM nicotine, as measured in $\beta 2^{-/-}$ mice (□) and WT mice continually administered saline (■) or nicotine (chron, □). ★★★, $P < 0.001$.

Index of Addiction in Humans. Ideal smoking cessation programs involve a combination of comprehensive medical, psychiatric, and substance-abuse evaluations. Current tests include the assessment of nicotine-dependence severity (e.g., Fagerström Test for Nicotine Dependence) and measurements of smoking and nicotine intake, including expired breath carbon monoxide and cotinine (a nicotine metabolite) levels in the plasma, saliva, or urine (Scherer and Richter, 1997; Benowitz, 1999). However, these routine methods are not fully satisfactory, because they are often not precise. Indeed, we found only a small correlation ($r < 0.3$) between the number of cigarettes smoked per day and the cotinine/creatinine ratio or the amounts of expired carbon monoxide in blood samples tested for the content of EB binding sites (data not shown). The evidence provided here that levels of EB binding sites in human blood PMN leukocytes can be correlated with tobacco consumption provides a novel and more accurate objective assay to measure tobacco addiction.

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