Pergamon

Biochemical Pharmacology, Vol. 50, No. 12, pp. 2001–2008, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02100-0

UP-REGULATION OF NICOTINIC ACETYLCHOLINE RECEPTORS FOLLOWING CHRONIC EXPOSURE OF RATS TO MAINSTREAM CIGARETTE SMOKE OR α4β2 RECEPTORS TO NICOTINE

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(*Received* 4 *April* 1995; *accepted* 21 *July* 1995)

Abstract—Smokers are reported to have a higher density of central nicotinic acetylcholine receptors (nAChRs) that non-smokers at autopsy. Whether this increased receptor density is a response to smoking or a result of genetic variability is not known. While sub-chronic treatment of rats and mice with nicotine results in up-regulation of central nAChRs, changes in receptor density in response to cigarette smoke have not been studied previously. In this study, male Sprague–Dawley rats were exposed nose-only for 13 weeks to mainstream cigarette smoke followed by assessment of [^{3}H]nicotine binding in five brain regions of smoke- and sham-exposed animals. In smoke-exposed animals, there was a significant increase in nAChR density in the cortex, striatum, and cerebellum (35, 25, and 31% increases, respectively), while there was no significant change in receptor density in the thalamus and hippocampus. Smoke exposure did not alter markedly the affinity of the receptor for nicotine in these brain regions. Furthermore, up-regulation of nAChRs did not alter the biphasic binding properties by which nicotine binds to its receptor. There were no changes in the association (fast phase) or isomerization (slow phase) rate constants, and the percent contribution of slow and fast phase binding to nAChRs was not altered in the up-regulated receptor population compared with control. Similar results were observed following chronic nicotine exposure of cultured cortical cells from fetal rat brain or cells transfected with the α4β2 nAChR subtype. These results show that the up-regulation following smoke exposure in the rat is phenomenologically similar to that observed *in vitro*. These data provide preliminary evidence for a relationship between cigarette smoking and nAChR up-regulation *in vivo* and suggest that similar mechanisms of upregulation may underlie chronic smoke exposure of live animals and nicotine exposure of artificially expressed α4β2 receptors *in vitro*.

Key words: nicotine; nicotinic receptors; cholinergic; smoking; up-regulation

Nicotine, acetylcholine, and cytisine saturably bind with high affinity to nAChRs§ in the central nervous systems of mice, rats, and humans, and in cultured primary rat cortical neurons [1–6]. It is generally accepted that receptor systems up- and down-regulate in response to sub-chronic exposure to antagonists and agonists, respectively [7]. In contrast, several studies have shown that in mice and in rats nAChRs up-regulate in response to sub-chronic exposure to nicotine [reviewed in Ref. 8]. In addition, in rats the α4 and β2 subunits, which are thought to comprise the high affinity receptor, are up-regulated to the same extent as the active nAChR complex in response to sub-chronic nicotine exposure [9]. Interestingly, in mice the increase in receptor density is not accompanied by an increase in α4 or β2 subunit mRNA [10]. This is also true in rats, where sub-chronic nicotine exposure does not increase hypothalamic α3 subunit mRNA [11]. Based on studies of primary cultures from fetal rat brain and M10 cells or *Xenopus* oocytes expressing α4β2 subunits [12], it appears that up-regulation is mediated exclusively through post-translational mechanisms.

The nAChR pool is also reported to vary within the human population. The density of nAChRs is higher in the brains of human smokers than in non-smokers [13], while nAChR density is reduced in various brain regions of humans diagnosed with Alzheimer's or Parkinson's disease [14–16]. It is not known whether the increased density of nAChRs in human smokers is a direct result of smoking or if a subset of the human population may have a higher density of central nAChRs. The latter possibility is suggested by the observation that inbred mouse strains show differences in the density of [^{3}H]-nicotine binding in many brain regions [17]. To evaluate the effects of exposure to mainstream tobacco smoke on the characteristics of [^{3}H]nicotine binding in various brain regions, we compared the results from a smoke exposure study with those obtained in *in vitro* systems.

We found that smoke exposure led to changes in nAChR density in rats that were similar to changes following nicotine exposure seen in primary cultures of fetal rat brain and α4β2 expressed in non-neuronal cells. Furthermore, this up-regulation was not associated with a change in the affinity of the nAChR for nicotine, nor was the up-regulation accompanied by changes in the binding kinetics of nicotine to the nAChR. A preliminary report of portions of this work has been presented [18].

MATERIALS AND METHODS

Animals and materials

Male Sprague–Dawley rats (Crl: CD/BR, VAF/Plus) were purchased from Charles River Laboratories (Ra-

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§ Abbreviations: nAChR, nicotinic acetylcholine receptor; DMEM, Dulbecco's Modified Eagle's Medium; and KRH, Krebs-Ringer HEPES.

leigh, NC) and housed one per cage on a 12-hr light/dark schedule with *ad lib.* access to Purina Rodent Chow 5002 (Purina Mills, Inc., Richmond, IN) and water in accordance with the Animal Welfare Act of 1970 and amendments. Animals were acclimated to laboratory conditions for 3 weeks prior to the first smoke exposure. L-(−)-[*N-methyl*-^{3}H]nicotine ([^{3}H]nicotine) (71–75 Ci/mmol) was purchased from the du Pont Company/NEN Research Products (Boston, MA). Free base nicotine (99% pure) was purchased from the Eastman Kodak Co. (Rochester, NY). All other chemicals were of reagent grade. Dexamethasone and carbachol were obtained from Sigma (St. Louis, MO); fetal serum, DMEM and DMEM/F12 from GIBCO, Grand Island, NY; and protein reagents from Pierce, Rockford, IL.

Animal exposure to mainstream cigarette smoke

The inhalation exposure system has been described in detail elsewhere [19, 20]. Analysis of the chemical composition of the cigarette smoke was performed on smoke generated with the standard FTC puffing regimen (Federal Trade Commission, 1967). Prior to exposure, cigarette smoke was diluted with HEPA-filtered air and humidified (40–60% relative humidity) at 20–24° to yield the following composition (mean ± SD): 0.64 ± 0.03 mg/L wet total particulate matter, 37 ± 2 μg/L nicotine, 773 ± 53 ppm CO. Immediately prior to exposure, animals were placed in conical restrainers to permit nose-only exposure to cigarette smoke. Animals were exposed to cigarette smoke for 1 hr per day, 5 days per week, for 13 consecutive weeks. This protocol was part of a larger study, and neither exposure amounts nor duration could be varied to study the effects on up-regulation. Sham operant control animals were treated in the same fashion, but were exposed only to HEPA-filtered, humidified air.

Determinations of plasma nicotine levels

Immediately following cigarette smoke exposure, blood was drawn from the retro-orbital sinus of anesthetized animals (70% CO_2 in air) using heparinized micropipettes. Samples were transferred to Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing Na_2-EDTA and immediately centrifuged. Plasma was collected and stored at −70° prior to analysis. Plasma nicotine concentrations were determined by a monoclonal antibody ELISA. The assay was performed based on the method of Bjercke *et al.* [21], as modified by Chang *et al.* [22]. In addition, a computerized pharmacokinetic model of nicotine disposition in the rat [23] was used to simulate plasma nicotine concentrations based on nicotine exposure in the cigarette smoke inhalation chamber. The pharmacokinetic parameters used in this simulation have been described previously [23] with the exception of minute ventilation, which was decreased by 70% (i.e., to 55 mL/min) in smoke-exposed animals compared with sham controls. Body weight was also decreased by 5%, and an average body weight of 281 g was used for smoke-exposed animals during the first 2 weeks of the study.

Tissue preparation

Animals were euthanized by first anesthetizing with 70% CO_2 in air followed by exsanguination. Brains were removed, dissected into regions (cerebral cortex, corpus striatum, hippocampus, thalamus, and cerebellum), and frozen in KRH buffer at −80° prior to binding experiments (2–4 months). The KRH buffer was composed of the following (mM): 118 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 20 HEPES, and 2.5 $CaCl_2$ (pH 7.5 at 4°). On, or just prior to, the day of binding experiments the tissue was thawed and homogenized on ice in 10 vol. of KRH buffer with 25 strokes of a motor-driven pestle at 860 rpm. The resultant homogenate was incubated at 37° for 5–10 min and centrifuged at 51,500 *g* for 20 min. The resultant pellet was suspended in ice-cold, distilled-deionized water, incubated on ice for 30 min, incubated at 37° for 5–10 min, and centrifuged as above. This lysis step was repeated, and the final pellet was resuspended in distilled-deionized water to yield the desired protein concentration (see below).

[^{3}H]Nicotine equilibrium binding

Equilibrium binding assays were conducted in 96-well assay plates (Corning, Corning, NY) in a total volume of 100 μL, as described by Romano and Goldstein [24] and modified by Marks *et al.* [25]. Saturable binding was measured in the cortex, striatum, hippocampus, thalamus, and cerebellum. A concentration range of 0.625 to 20 nM [^{3}M]nicotine was used in the presence or absence of 10 μM unlabeled nicotine to account for non-specific binding (blanks). Each batch of newly purchased [^{3}H]-nicotine was purified by two-dimensional thin-layer chromatography as previously described [26] prior to use. Assays were initiated by the addition of 40–120 μg protein/well and incubated on ice in a 4° room for 2 hr. Total binding for each concentration was determined in quintuplicate; blanks were determined in triplicate. Assays were terminated by dilution and rapid filtration (five washes) using an Inotech Cell Harvester (Inotech Biosystems International, Lansing, MI) equipped with 1 thickness of Gelman (Ann Arbor, MI) type A/E glass fiber filter and 1 thickness of Micro Filtration Systems (Dublin, CA) type GB100RN glass fiber prefilter. Both filters were presoaked in KRH buffer containing 0.5% (w/v) polyethyleneimine prior to use to reduce non-specific binding of [^{3}H]nicotine. Filters were collected, placed in Packard miniature vials (Packard Instrument Co., Meriden, CT), agitated with 3 mL of EcoLite(+) liquid scintillation solution for 15 min (ICN Biomedicals, Irvine, CA), and counted in a Packard model 2000CA liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL).

Primary cultures of fetal rat brain

Sprague–Dawley timed pregnant rats purchased from Harlan Industries (Indianapolis, IN) were maintained on a 12-hr light/dark cycle with water and standard Purina Rat Chow *ad lib.* On day 17 of gestation, dams were killed using CO_2 anesthesia followed by decapitation, and rat fetuses were removed from the uterus. Following decapitation and opening of the cranium, the cerebellum was removed from each fetus and cerebral cortices were recovered and processed according to previously described protocols [27]. Briefly, tissue was placed in ice-cold PBS containing 0.5 g/L glucose (pH 7.4), and the cells were dissociated mechanically by successive passage through a Nitex bag from Tetko Inc. (210 μm pore size), a metal sieve (180 μm), and another Nitex bag (30 μm). The preparation was then centrifuged, and the resulting pellet was diluted to achieve an initial plating density of approximately 2 million cells/100 mm tissue culture dish. Cells were incubated at 37° in 5% CO_2 in

DMEM/F12 (from GIBCO) containing 10% fetal bovine serum. After 1 day, cells were grown for an additional 6 days in DMEM/F12 serum-free medium supplemented with 2 μM insulin, 0.2 mM putrescine, 0.2 g/L transferrin, 60 nM sodium selenite, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Mouse M10 clonal cells

The M10 cell line is a mouse fibroblast clone stably transfected with chick cDNA corresponding to the subunits of the predominant brain nicotinic receptor (provided by Dr. Paul Whiting). Expression of the chick α4 and β2 subunit genes is under control of a steroid-inducible promoter and is from a vector that also contains an antibiotic-resistance gene for positive selection of transfected cells. Cells were grown in DMEM containing 10% fetal bovine serum according to standard protocols [28], except that the antibiotic geneticin (1 mg/mL) was added routinely to the medium to eliminate any revertants [29]. Our studies indicate that, in the presence of dexamethasone, M10 cells exhibit a high affinity nicotine binding site with binding characteristics similar to those expressed in mammalian brain (see below).

[^{3}H]Nicotine association kinetics

Rat brain. Association binding assays were conducted in 48-well tissue culture plates (Costar, Cambridge, MA) in a total volume of 100 μL as described above. The kinetics of association binding were determined only in cortical tissue, as previously described [27]. A constant concentration of [^{3}H]nicotine (11 nM) was used in the presence or absence of 10 μM unlabeled nicotine to account for non-specific binding (blanks). Assays were initiated by the addition of 100–200 μg protein/well and incubated on ice in a 4° room for 0.5–120 min. Total binding for each time point was determined in quadruplicate, with single blank determinations at each time point. Assays were terminated by dilution and rapid filtration (five washes in approximately 20 sec), and radioactivity was quantitated as described above.

Cells. Cells were mechanically scraped, harvested in cold Tris buffer (5 mM), and homogenized with a Polytron (from Brinkmann Instruments, Westbury, NY; settings at full power for 10 sec). The homogenate was centrifuged at 40,000 *g* for 10 min, the supernatant was discarded, and the pellet was reconstituted in PBS (pH 7.4). Standard procedures for ligand binding studies were followed, and, routinely, sample aliquots were reserved for determination of protein concentration [30] with bovine serum albumin as the standard. Equilibrium binding assays were conducted by incubating membrane aliquots suspended in 300 μL assay buffer with [^{3}H]-nicotine (Dupont, NEN). Saturation binding studies were conducted on 48-well plates using at least eight different concentrations of [^{3}H]nicotine ranging from 1 to 20 nM for 2 hr. Association kinetics studies were conducted using a single concentration of [^{3}H]nicotine (10 nM) for times ranging from 1 to 120 min. Non-specific binding was determined by addition of 10 μM nicotine or 1 mM carbachol. Incubation was terminated by rapid filtration on a multimanifold tissue harvester (Brandel) using G/C filters presoaked in polyethyleneimine. All binding experiments were performed at 4°. Concentration of receptor and dissociation constant were determined by Scatchard analysis of the saturation binding data. Fast and slow rate constants for [^{3}H]nicotine binding were extrapolated from logarithmic transformation of the association kinetics on membrane preparations from naive M10 or cells pretreated with 10 μM nicotine.

Data and statistical analyses

B_{max} and K_d were determined by Scatchard analysis (plot of bound ligand/free ligand vs bound ligand), where K_d is equal to the negative inverse of the slope and B_{max} is equal to the x-intercept. Association kinetics were determined as previously described [27], where the binding at equilibrium (B_e) is equivalent to the maximum nicotine binding achieved with a given sub-saturating concentration of [^{3}H]nicotine, the association rate constant (k_{obs}) is determined from the initial rapid phase of nicotine binding, and the isomerization constant is determined from the slower second phase of nicotine binding. All values are presented as means ± SEM with N = 6–7 for equilibrium binding and association kinetics, where one N is equivalent to a given brain region from a single animal (i.e. tissues were not pooled). Thus, the variability among means reflects variation between animals as well as variation among preparations and experiments. Data were analyzed by *t* test, and the calculated *P* values are provided. A *P* value of <0.05 was taken as the level of significance for all comparisons.

RESULTS

Plasma nicotine levels

Exposure of animals to cigarette smoke for 1 hr resulted in a 25-fold increase in plasma nicotine as determined by ELISA (Fig. 1). The experimental protocol for the exposure of animals to cigarette smoke prevented blood sampling at more than one time point (t = 1 hr) following smoke exposure. Thus, a pharmacokinetic model, previously validated for nicotine exposure in rats,

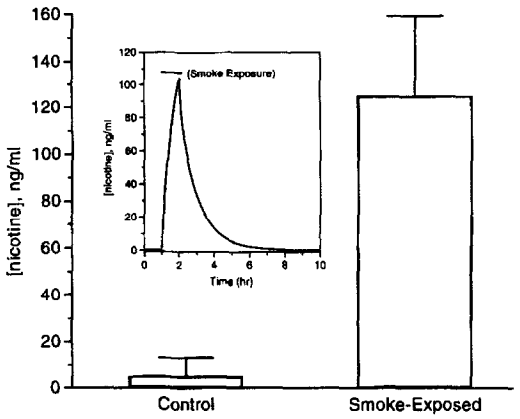

Fig. 1. Plasma nicotine concentrations following cigarette smoke exposure. Plasma was collected immediately following the termination of cigarette smoke exposure, and nicotine concentrations were determined by ELISA for smoke-exposed and sham operant animals (means ± SEM, N = 45–49). The inset shows a simulation from a pharmacokinetic model of nicotine disposition in the rat [23]. Plasma concentrations of nicotine were simulated using a 1-hr inhalation exposure to cigarette smoke during week number 1 of the 13-week study.

was used to estimate the profile of plasma nicotine from the onset of one exposure through the onset of a subsequent exposure [23]. The results of a 24-hr computerized simulation are shown in the inset to Fig. 1. The concentration of plasma nicotine rose rapidly during the 1-hr smoke exposure and decayed exponentially upon termination of exposure. Plasma nicotine levels reached background within 6 hr following termination of smoke exposure. The pharmacokinetic model also predicted that over a 5-day exposure there would be no accumulation of nicotine in the plasma (data not shown). The peak plasma concentration of nicotine predicted by the model (*ca.* 105 ng/mL) was in good agreement with the actual plasma values (*ca.* 125 ng/mL) determined by ELISA (see Fig. 1).

Binding characteristics of brain nAChR from in vivo *studies*

The equilibrium binding properties of nAChRs in control and smoke-exposed animals were studied in five brain regions. A representative [^{3}H]nicotine binding saturation curve is shown in Fig. 2 for striatal tissue from control and smoke-exposed rats. Scatchard analysis (insets to Fig. 2) depicts a single class of binding sites. K_d (−1/slope) values ranged from 3.5 to 4.5 nM, and B_{max} (x-intercept) ranged from 50 to 70 fmol/mg protein for control and smoke-exposed tissues, respectively.

The K_d and B_{max} values for each brain region are shown in Fig. 3. Both the K_d and B_{max} values tended to be increased as a result of smoke exposure. Statistically significant increases in B_{max}, however, were observed only for striatum, cortex, and cerebellum, while both the B_{max} and K_d were increased significantly in the striatum in response to cigarette smoke exposure. Thus, the increase in [^{3}H]nicotine binding appeared to result primarily from an increase in the B_{max} and not from an alteration in the affinity of binding, since there were no significant differences (except in the striatum) in the K_d values between control and smoke-exposed groups.

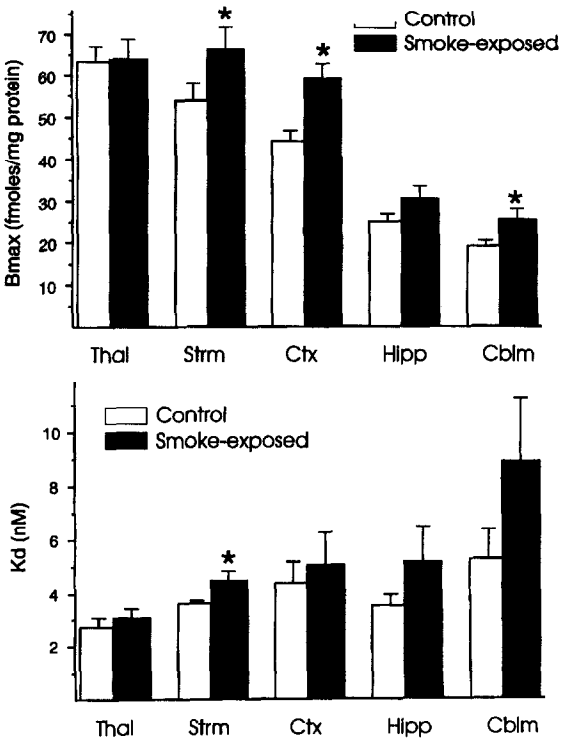

Fig. 3. B_{max} and K_d values for various brain regions. B_{max} and K_d values were estimated from Scatchard analyses for five brain regions: thalamus (Thal), striatum (Strm), cortex (Ctx), hippocampus (Hipp) and cerebellum (Cblm), for control and smoke-exposed rats. Asterisks represent parameters significantly different between the two groups ($P < 0.05$). Values are means ± SEM (N = 6–7).

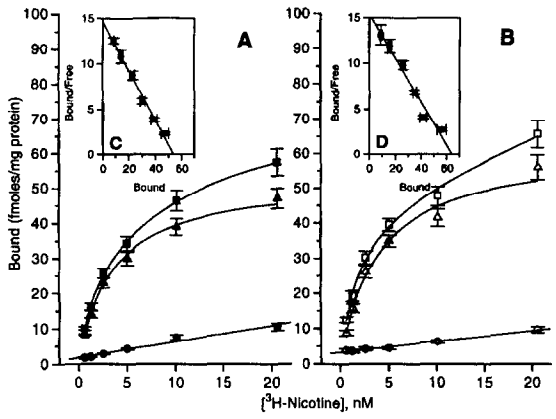

Fig. 2. Binding of [^{3}H]nicotine to rat striatal membranes. Saturation binding of nicotine was determined for membranes prepared from rat striatum of control (A) and smoke-exposed (B) animals. Membranes were incubated for 2 hr at 4° with 0.625 to 20 nM [^{3}H]nicotine to determine total binding (A, closed square or B, open square) and in the presence of 20 μM unlabeled nicotine to determine non-specific binding (A, closed circle or B, open circle). Specific binding (A, closed triangle or B, open triangle) was calculated from the difference between total and non-specific binding. Insets: Scatchard analysis of [^{3}H]nicotine binding to membranes prepared from rat striatum of control (C) and smoke-exposed (D) animals. All data are expressed as means ± SEM, N = 7.

The time-course of nicotine binding was analyzed to assess the kinetics of [^{3}H]nicotine binding. The association kinetics for [^{3}H]nicotine binding in the cortex from control and smoke-exposed animals are shown in Fig. 4. Consistent with the results reported above, smoke exposure resulted in an up-regulation of nAChRs as evidenced by the increase in the amount of nicotine bound at equilibrium (B_e) for 11 nM [^{3}H]nicotine. B_e increased by 22% from 41.6 ± 3.7 fmol/mg protein in controls to 50.3 ± 6.8* fmol/mg protein in smoke-exposed animals (mean ± SEM, N = 6, *$P = 0.02$). Up-regulation of the nAChR, however, did not alter the kinetics of nicotine binding. Neither the rates of association (k_{obs}, initial rapid phase binding) or isomerization (k_s, secondary slow phase binding), nor the percent of slow phase binding (B_s) were altered in the up-regulated receptor population (see Table 1).

Binding characteristics of brain nAChRs from in vitro *studies*

Following plating for several days, M10 cells exposed to dexamethasone (1 μM) for 2–3 days expressed a single population of high affinity nicotine binding sites (K_d = 2.5 nM; B_{max} = 210 fmol/mg protein). No significant

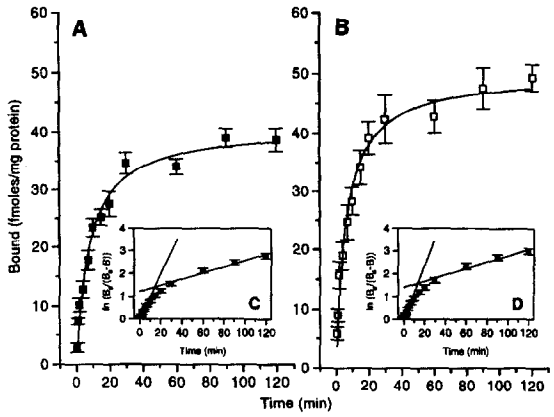

Fig. 4. Kinetics of [^{3}H]nicotine binding to rat cortical membranes. Association binding of nicotine was determined for membranes prepared from rat striatum of control (A) and smoke-exposed (B) animals. Membranes were incubated for 0.5–120 min at 4° with 11 nM [^{3}H]nicotine to determine total binding. Specific binding (A, closed squares or B, open squares) was calculated from the difference between total and non-specific binding (binding in the presence of 10 μM unlabeled nicotine). Insets: kinetic analysis of [^{3}H]nicotine binding to membranes prepared from rat striatum of control (C) and smoke-exposed (D) animals. The rates of association (k_{obs}) and isomerization (k_s), as well as the binding equilibrium (B_e) for 11 nM nicotine and the percent slow phase binding were determined graphically (see Table 1). Values are means ± SEM (N = 6–7).

specific high affinity binding was observed in membrane preparations from cells when dexamethasone was omitted, or in the presence of nicotine alone. Exposure of steroid-pretreated M10 cells to 1 μM nicotine for 1 day resulted in a marked increase in high affinity nicotine binding site density to 260% of control values (Table 1). These effects were apparent after 1 day but not after a 1-hr exposure to nicotine and were concentration dependent from 10 nM to 10 μM (EC_{50} = 100 nM). Scatchard analyses of binding data indicated that exposure to nicotine resulted in an increase in receptor number (B_{max}) with no significant change in affinity of the ligand (K_d) for the receptor (Table 1).

Association kinetic studies also were performed on membrane fractions derived from control or nicotine-treated M10 cells. Aliquots were incubated with 10 nM [^{3}H]nicotine for various time periods from 1 to 120 min. Non-specific binding was determined by the addition of 10 μM nicotine. The logarithmic transformation of the binding data indicated two components in the kinetics of association: a fast phase (k_{obs} = 0.2/min^{-1}; 45% of the total binding) and a slow isomerization phase (k_s = 0.02/min^{-1}; 55% of the total binding). These parameters were not changed significantly following nicotine treatment, similar to results obtained from rats exposed to smoke (summarized in Table 1).

Additional experiments using cultured cortical cells from fetal rat brain indicated a pattern of up-regulation following nicotine exposure similar to that seen both in smoke-exposed animals and M10 cells. Scatchard analyses of saturation binding isotherms indicated a single population of high affinity sites (K_d = 2.4 nM and B_{max} = 12 fmol/mg protein) in primary cultures. Following chronic nicotine treatment (1 μM for 5 days), the density of high affinity binding sites increased to 261 ± 8% of control levels, remarkably similar to the results from M10 cells (Fig. 5).

DISCUSSION

The observation that sub-chronic nicotine exposure results in an increased density of nAChRs in various brain regions is well established in the rat and mouse [reviewed in Ref. 8]. In such studies, nicotine was administered either by continuous infusion or periodic injection. These routes, however, are not representative of the major route of human exposure, that being the inhalation of tobacco smoke. The inhalation exposure regimen in this study did result in a significant increase in [^{3}H]nicotine binding in the cortex, striatum, and cerebellum, while there were no observed changes in binding in the thalamus and hippocampus (Figs. 2 and 3). Although the changes in receptor density were statistically significant in only three brain regions, the only brain region that did not show a trend toward increased density was the thalamus. The pattern of up-regulation in the present studies is different from that previously shown in humans [13], in that individuals who smoke tobacco products appear to have an increased density of nAChRs in several gray matter brain regions. In this study, rats were exposed to cigarette smoke containing 37 μg/L nicotine, resulting in peak plasma nicotine values of 125 ng/mL after a 1-hr exposure (Fig. 1). In smokers, blood or plasma concentrations of nicotine are in the general range of 10–50 ng/mL [31]. Taking into account that a rat metabolizes nicotine at a rate 4-fold faster than the human [32], the amount of nicotine inhaled by the rat in the present study was between 8 and 40 times greater than that received by the average human smoker. It is, however, difficult to compare the rat exposure in this study with typical human exposure since the rats were exposed for only 1 hr and human smokers are exposed for various periods throughout the day.

The inhalation exposure regimen resulted in increased [^{3}H]nicotine binding in several brain areas. It is clear that the increase in binding is a direct result of increased receptor density, since there was no change in the affinity of the receptor for nicotine. Exposure of rats to cigarette smoke resulted in a 35% increase in the B_{max} of nAChRs in rat cortex. This result is strikingly similar to those of Schwartz and Kellar [33], who observed a 31% increase in the B_{max} and no change in the K_d of nAChRs in the cortex of rats treated with 1 mg/kg nicotine twice daily for 10 days. Flores *et al.* [9] have also reported that the α4 and β2 subunits, which are thought to compose the high affinity nAChR, are increased by a similar percentage in response to periodic nicotine injections, as described by Schwartz and Kellar [33]. Thus, it is likely that the increase in nAChR density observed in the present study is a result of the nicotine content of tobacco smoke. However, we cannot rule out the actions of other known or potential nicotinic agonists found in tobacco smoke, such as anabasine, which has been suggested to play a role in the up-regulation process [34].

Based on binding experiments using neuronal tissue and primary cortical neurons in culture, the nAChR may exist in two confirmations. Lippiello *et al.* [27] have shown that the association of [^{3}H]nicotine to the nAChR

Table 1. Comparison of binding and kinetic parameters for rat brain following smoke exposure and cultured cells following nicotine exposure

	K_d (nM)	B_{max} (fmol/mg)	B_s (%)	k_{obs} (nM^{-1} min^{-1})	k_s (min^{-1})
Rat brain cortex					
Control	4.45 ± 0.79	44.5 ± 2.0	30.0 ± 7.6	0.097 ± 0.022	0.013 ± 0.001
Smoke-exposed	5.12 ± 1.15	59.5 ± 3.5*	25.5 ± 6.7	0.116 ± 0.030	0.013 ± 0.001
M10 cells					
Control	2.5 ± 0.5	210 ± 15	42 ± 15	0.200 ± 0.10	0.020 ± 0.01
Nicotine	2.0 ± 0.4	546 ± 86†	45 ± 7	0.08 ± 0.006	0.024 ± 0.011

Membranes were prepared from control and smoke-exposed animals or M10 cells following control and nicotine treatment (see Materials and Methods). Equilibrium binding and binding kinetics were carried out as described in Materials and Methods. Equilibrium binding parameters (K_d and B_{max}) were derived from Scatchard analysis of binding data. The fast rate of association (k_{obs}) and slow rate of isomerization (k_s) as well as the percent slow phase binding (B_s) for [^{3}H]nicotine were determined graphically. All data are expressed as means ± SEM (N = 6 for smoke-exposure studies, N = 11 for M10 cells).

* $P < 0.05$.

† $P < 0.002$.

is biphasic, where there is an initial rapid binding phase to a high affinity receptor population (association rate k_{obs}), followed by a slower binding phase that most likely represents a conformational change (isomerization rate k_s) in other receptors from a low affinity state to the high affinity receptor state. At sub-saturating nicotine concentrations, roughly 60% of the binding is to the pre-existing high affinity receptors, while 40% of the binding occurs by a slow phase process representing isomerization of receptors to the high affinity state [4, 27]. The results reported here suggest that the up-regulated population of receptors has the same binding kinetics profile (k_{obs} and k_s) as control nAChR populations (Fig. 4). There was, however, a significant increase in the equilibrium binding (B_e) for [^{3}H]nicotine (maximum binding at sub-saturating concentrations of ligand). This 25% increase in B_e is in agreement with the increase in B_{max} observed in the rat cortex (see discussion above). We also found that the population distribution for the two states of the nAChR at sub-saturating nicotine concentrations was not altered in the up-regulated receptor population. Accordingly, approximately 30% of the receptors bound ligand by the slow phase process; thus, around 70% of the binding was to receptors in the high affinity state (Fig. 4), versus 40 and 60%, respectively, in rat brain at similar nicotine concentrations [27]. Similar results have been reported in mouse brain [35]. We cannot rule out the possibility that the new nAChRs in the up-regulated population do have altered [^{3}H]nicotine binding kinetics, but these changes cannot be distinguished in a pool of new and previously existing receptors. It is likewise possible that the time elapsed between killing the animal and isolating the tissue to perform binding assays was long enough to prevent readily reversible changes in binding kinetics from being detected, or that the process of receptor isolation may have altered the proportion of low and high affinity nAChR populations from their *in vivo* proportions.

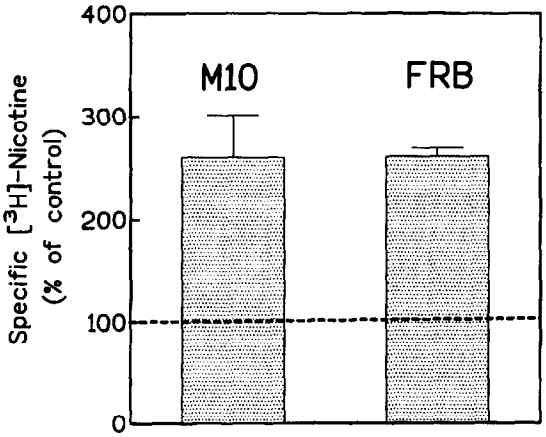

Fig. 5. Histograms of specific [^{3}H]nicotine binding to M10 cells and primary cultures of fetal rat brain (FRB) expressed as percent per mg protein of control values. Membranes were prepared according to protocols described in Materials and Methods. In the absence of dexamethasone, no detectable high affinity binding sites were present with or without nicotine. Dotted line: Equilibrium receptor number after treatment in the presence of dexamethasone (1 μM) for 2 days (100%). M10: Increased high affinity binding in M10 cells after treatment with 1 μM nicotine for an additional 24 hr (260 ± 40%; $P < 0.002$ from N = 11). FRB: Increased high affinity binding in fetal rat brain cells after treatment with 1 μM nicotine for an additional 5 days (261 ± 8%; N = 3). Control values were 210 fmol/mg protein in M10 cells and 12 fmol/mg protein in fetal rat brain. All values are means ± SEM.

Since smoke exposure results in an increased density of nAChRs and the percent distribution of receptor states remains unchanged (Fig. 4), our observations suggest that in the up-regulated population of nAChRs there would be a greater total number of activatable receptors compared with control receptor populations. Thus, with more activatable receptors it would be expected that there would be greater functional capacity for nAChR-mediated neuronal activities. We did not examine functional end points in the up-regulated receptor populations in the present studies, but a number of other workers have reported conflicting results with regard to the function of up-regulated receptors. For example, Rowell and Wonnacott [36] reported that, as a result of nAChR up-regulation by sub-chronic exposure to the nicotinic agonist anatoxin-a, there was a 32% increase in the B_{max} of rat striatal synaptosomes associated with a 43% increase in nicotine-stimulated [^{3}H]dopamine release.

However, Harsing *et al.* [37] found that sub-chronic treatment of mice with nicotine did not enhance nicotine-induced [^{3}H]dopamine release from superfused whole striatum. In some cases, for example nicotine-induced acetylcholine release from rat hippocampal slices [38] and dopamine release from mouse striatal synaptosomes [10], receptor function actually seemed to be diminished in animals treated sub-chronically with nicotine, compared with untreated animals. The latter studies also showed that this decreased activity persisted for several days after the termination of nicotine treatment, a period longer than the time required for up-regulated receptors to begin returning to control densities. Thus, although the up-regulated population of nAChRs appears to bind nicotine in the same fashion as the control population, the functional activities of the up-regulated receptor population are in question. Some of the discrepancies mentioned above may be explained by recent studies suggesting that nicotine-stimulated dopamine release in striatum may result from activation of α-3 containing receptors rather than the α-4 containing receptor believed to represent the high affinity nicotine binding sites [39]. Receptors containing the latter subunit have been suggested to mediate isotopic ion flux measured in mouse midbrain [40] and show complex kinetics for the onset and offset of desensitization [41]. Furthermore, it is not yet known if nAChRs that are up-regulated by cigarette smoke will show the same reduction in functional activities as receptors that are up-regulated as a result of sub-chronic nicotine exposure. Clearly this is an area that will require further studies to resolve the incongruent relationship between nicotine receptor density and receptor function.

In conclusion, the present study demonstrated that sub-chronic exposure of rats to cigarette smoke results in increased [^{3}H]nicotine binding in cortex, striatum, and cerebellum. This increase in binding was a result of increased nAChR density since there was no change in the affinity of the receptor for nicotine. The phenomenologically similar results obtained following chronic nicotine exposure of fetal rat brain neurons in culture and in non-neuronal cells expressing α4 and β2 subunit genes strongly suggest that effects observed *in vivo* result from nicotine effects and that these effects are (i) not mediated through indirect mechanisms resulting from CNS cytoarchitecture since similar results are observed under culture conditions, and (ii) not the result of the neuronal features of the cells expressing these receptors but rather an intrinsic attribute of the receptor protein. Our findings that cigarette smoke exposure does increase nAChR density in rats suggests that the increased density of these receptors found postmortem in human smokers [13] is most likely a result of smoking and not genetic differences in receptor densities in the brain. Additional studies are required to determine the functional capacity of these up-regulated receptors and to determine the relationships between receptor up-regulation, smoking, and the occurrence of neurodegenerative diseases.

REFERENCES

1. Marks MJ and Collins AC, Characterization of nicotine binding in mouse brain and comparison with the binding of α-bungarotoxin and quinuclidinyl benzilate. *Mol Pharmacol* **22:** 554–564, 1982.
2. Schwartz RD, McGee R Jr and Kellar KJ, Nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine in rat brain. *Mol Pharmacol* **22:** 56–62, 1982.
3. Lippiello PM and Fernandes KG, The binding of L-[^{3}H]-nicotine to a single class of high affinity sites in rat brain membranes. *Mol Pharmacol* **29:** 448–454, 1986.
4. Lippiello PM and Fernandes KG, Identification of putative high affinity nicotine receptors on cultured cortical neurons. *J Pharmacol Exp Ther* **246:** 409–416, 1988.
5. Pabreza LA, Dhawan S and Kellar KJ, [^{3}H]Cytisine binding to nicotinic cholinergic receptors in brain. *Mol Pharmacol* **39:** 9–12, 1991.
6. Hall M, Zerbe L, Leonard S and Freedman R, Characterization of [^{3}H]cytisine binding to human brain membrane preparations. *Brain Res* **600:** 127–133, 1993.
7. Klein WL, Sullivan J, Skorupa A and Aguilar JS, Plasticity of neuronal receptors. *FASEB J* **3:** 2132–2140, 1989.
8. Wonnacott S, The paradox of nicotinic acetylcholine receptor upregulation by nicotine. *Trends Pharmacol Sci* **11:** 216–219, 1990.
9. Flores CM, Rogers SW, Pabreza LA, Wolfe BB and Kellar KJ, A subtype of nicotinic cholinergic receptor in rat brain is composed of α4 and β2 subunits and is up-regulated by chronic nicotine treatment. *Mol Pharmacol* **41:** 31–37, 1992.
10. Marks MJ, Pauley JR, Gross SD, Deneris ES, Hermans-Borgmeyer I, Heinemann SF and Collins AC, Nicotine binding and nicotine receptor subunit RNA after chronic nicotine treatment. *J Neurosci* **12:** 2765–2784, 1992.
11. Hollt V and Horn G, Effect of nicotine on mRNA levels encoding opioid peptides, vasopressin and α3 nicotinic receptor subunit in the rat. *Clin Investig* **70:** 224–231, 1992.
12. Peng X, Gerzanich V, Anand R, Whiting PJ and Lindstrom J, Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol* **46:** 523–530, 1994.
13. Benwell MEM, Balfour DJK and Anderson JM, Evidence that tobacco smoking increases the density of (–)-[^{3}H]nicotine binding sites in human brain. *J Neurochem* **50:** 1243–1247, 1988.
14. Whitehouse PJ, Martino AM, Marcus KA, Zweig RM, Singer HS, Price DL and Kellar KJ, Reductions in acetylcholine and nicotine binding in several degenerative diseases. *Arch Neurol* **45:** 722–724, 1988.
15. Giacobini E, Cholinergic receptors in human brain: Effects of aging and Alzheimer's disease. *J Neurosci Res* **27:** 548–560, 1990.
16. Rinne JO, Myllykyla T, Lonnberg P and Marjamaki P, A postmortem study of brain nicotinic receptors in Parkinson's and Alzheimer's disease. *Brain Res* **547:** 167–170, 1991.
17. Marks MJ, Stitzel JA and Collins AC, Genetic influences on nicotinic responses. *Pharmacol Biochem Behav* **33:** 667–678, 1989.
18. Yates SL, Fernandes KG, Fluhler EN and Lippiello PM, Regional changes in rat central nicotinic acetylcholine receptors following nose-only exposure to mainstream cigarette smoke. *Soc Neurosci Abstr* **18:** 816, 1992.
19. Coggins CRE, Ayres PH, Mosberg AT, Sagartz JW, Burger GT and Hayes AW, Ninety-day inhalation study in rats, comparing smoke from cigarettes that heat tobacco with those that burn tobacco. *Fundam Appl Toxicol* **13:** 460–483, 1989.
20. Ayres PH, Mosberg AT and Coggins CRE, Modernization of nose-only smoking machines for use in animal inhalation studies. *J Am Coll Toxicol* **9:** 441–446, 1990.
21. Bjercke RJ, Cook G, Rycklik N, Gjika HB, Van Vunakis H and Langone JJ, Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assays. *J Immunol Methods* **90:** 203–213, 1986.
22. Chang K-M, Gentry G, Davis R, Stiles M and Coggins C,

Determination of plasma nicotine and cotinine in rats exposed to aged and diluted side stream smoke, using an enzyme-linked immunosorbent assay (ELISA). *Toxicologist* **12:** 263, 1992.
23. Plowchalk DR, Anderson ME and deBethizy JD, A physiologically based pharmacokinetic model for nicotine disposition in the Sprague-Dawley rat. *Toxicol Appl Pharmacol* **116:** 177–188, 1992.
24. Romano C and Goldstein A, Stereospecific nicotine receptors on rat brain membranes. *Science* **210:** 647–649, 1980.
25. Marks MJ, Stitzel JA, Romm E, Wehner JM and Collins AC, Nicotinic binding sites in rat and mouse brain: Comparison of acetylcholine, nicotine, and α-bungarotoxin. *Mol Pharmacol* **30:** 427–436, 1986.
26. Romm E, Lippiello PM, Marks MJ and Collins AC, Purification of L-[^{3}H]nicotine eliminates low affinity binding. *Life Sci* **46:** 935–943, 1990.
27. Lippiello PM, Sears SB and Fernandes KG, Kinetics and mechanism of L-[^{3}H]nicotine binding to putative high affinity receptor sites in rat brain. *Mol Pharmacol* **31:** 392–400, 1987.
28. Bencherif M and Lukas RJ, Differential regulation of nicotinic acetylcholine receptor expression by human TE671/RD cells following second messenger modulation and sodium butyrate treatments. *Mol Cell Neurosci* **2:** 52–65, 1991.
29. Whiting PJ, Schoepfer R, Lindstrom JM and Priestley T, Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. *Mol Pharmacol* **40:** 463–472, 1991.
30. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–255, 1976.
31. Benowitz NL, Porchet H and Jacob P, Pharmacokinetics and metabolism and pharmacodynamics of nicotine. In: *Nicotine Psychopharmacology: Molecular, Cellular and Behavioral Aspects* (Eds. Wonnacott S, Russell MAH and Stolerman IP), pp. 112–157. Oxford University Press, Oxford, 1990.
32. Plowchalk DR and deBethizy JD, Interspecies scaling of nicotine concentrations in the brain. In: *The Biology of Nicotine: Current Research Issues* (Eds. Lippiello PM, Collins AC, Gray JA and Robinson JH), pp. 55–70. Raven Press, New York, 1992.
33. Schwartz RD and Kellar KJ, Nicotinic cholinergic receptor binding sites in the brain: Regulation *in vivo*. *Science* **220:** 214–216, 1983.
34. Bhat RV, Turner SL, Selvaag SR, Marks MJ and Collins AC, Regulation of brain nicotinic receptors by chronic agonist infusion. *J Neurochem* **56:** 1932–1939, 1991.
35. Bhat RV, Marks MJ and Collins AC, Effects of chronic nicotine infusion on kinetics of high affinity nicotine binding. *J Neurochem* **62:** 574–581, 1994.
36. Rowell PP and Wonnacott S, Evidence for functional activity of up-regulated nicotine binding sites in rat striatal synaptosomes. *J Neurochem* **55:** 2105–2110, 1990.
37. Harsing LG Jr, Sershen H and Lajtha A, Dopamine efflux from striatum after chronic nicotine: Evidence for autoreceptor desensitization. *J Neurochem* **59:** 48–54, 1992.
38. Lapchak PA, Araujo DM, Quirion R and Collier B, Effect of chronic nicotine treatment on nicotine autoreceptor function and *N*-[^{3}H]methylcarbamylcholine binding sites in the rat brain. *J Neurochem* **52:** 483–491, 1989.
39. Grady S, Marks MJ, Wonnacott S and Collins AC, Characterization of nicotinic receptor-mediated [^{3}H]dopamine release from synaptosomes prepared from mouse striatum. *J Neurochem* **59:** 848–856, 1992.
40. Marks MJ, Farnham DA, Grady SR and Collins AC, Nicotinic receptor function determined by stimulation of rubidium efflux from mouse brain synaptosomes. *J Pharmacol Exp Ther* **264:** 542–552, 1993.
41. Marks MJ, Grady SR, Yang J-M, Lippiello PM and Collins AC, Desensitization of nicotine-stimulated $^{86}Rb^+$ efflux from mouse brain synaptosomes. *J Neurochem* **63:** 2125–2135, 1994.