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Downregulation of Nicotinic Receptor Function after Chronic Nicotine Infusion[1]

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ABSTRACT

Chronic nicotine treatment generally results in tolerance to several actions of nicotine and a paradoxical increase in brain nicotinic receptor numbers. Receptor upregulation, it has been argued, arises as a consequence of functional desensitization. In the studies reported here, mice were chronically infused with saline (control) or one of five doses of nicotine (0.25–4.0 mg/kg/hr) for 10 days. This treatment resulted in a dose-dependent tolerance to nicotine-induced decreases in body temperature as well as decreases in locomotor and rearing activities in a Y-maze. The anticipated increase in [^{3}H]nicotine binding was also observed. To assess functional status of the nicotinic receptors, nicotine-stimulated release of [^{3}H]dopamine from striatal synaptosomes and $^{86}Rb^+$ efflux from cortical and midbrain synaptosomes were also measured. Chronic nicotine infusion resulted in an infusion dose-dependent decrease in [^{3}H]dopamine release from striatum and $^{86}Rb^+$ efflux from midbrain; cortical $^{86}Rb^+$ efflux was not affected by chronic nicotine treatment. Dose-response analyses of the release and efflux assays demonstrated that chronic nicotine infusion evoked decreases in the maximal effects of nicotine on the functional assays; potency was not altered by chronic drug treatment. These results are consistent with the hypothesis that behavioral tolerance to nicotine is a consequence of down-regulation of brain nicotinic receptor function.

Animals treated chronically with nicotine almost invariably show changes in behavioral and physiological responses produced by acute injections of the drug. For example, several studies from our laboratory (Marks *et al.*, 1983, 1985b, 1986a,b, 1991) have demonstrated that mice that have been chronically infused with nicotine are tolerant to the effects of nicotine on locomotor activity, respiratory rate, heart rate, body temperature and acoustic startle response. Acute challenge doses of nicotine stimulate some of these measures (respiration and, in some mouse strains, acoustic startle), but depress others (locomotor activity, heart rate, body temperature). Chronic intravenous nicotine infusion results in tolerance to all of these measures as demonstrated by shifts to the right of dose-response curves. In addition, high doses of nicotine will elicit convulsions, and mice that have been chronically infused with nicotine show decreased sensitivity to nicotine's seizure-inducing effects (Miner and Collins, 1988). Similarly, rats that have been chronically injected, intraperioneally, or infused, subcutaneously, with nicotine show tolerance to the locomotor activity and body temperature depressant effects that are seen shortly after injection with high doses (>0.5 mg/kg) of nicotine (Collins *et al.*, 1988, 1990). Drug-naive rats generally show decreases in locomotor activity shortly after injection with nicotine, but this is often replaced by increases in activity that may persist up to an hour or more after injection (Clarke and Kumar, 1983). Rats that have been chronically injected with nicotine frequently exhibit increases in locomotor activity after low dose nicotine challenge (Clarke and Kumar, 1983; Ksir *et al.*, 1985, 1987; Clarke *et al.*, 1988; Fung and Lau, 1988; Shoaib and Stolerman, 1992; Benwell and Balfour, 1992).

The chronic administration of nicotine often causes an increase in the number of putative receptor sites measured by high-affinity agonist binding in mouse and rat brain (see the review by Wonnacott, 1990). Human smokers also show elevations in brain nicotinic receptor binding when compared to nonsmokers (Benwell *et al.*, 1988). Although early studies suggested that this up-regulation of brain nicotinic receptors might be associated with tolerance to nicotine in the mouse (Marks *et al.*, 1983, 1985b) more recent studies indicate that up-regulation can occur before tolerance development (Marks *et al.*, 1991) and tolerance can develop without receptor changes (Pauly *et al.*, 1992). Similarly, studies with the rat have shown that tolerance to nicotine's locomotor depressant effects is not inextricably associated with receptor changes (Collins *et al.*, 1990). Those studies that have detected sensitization to nicotine's locomotor activating effects have generally also detected increases in nicotinic receptor binding (Ksir *et al.*, 1985, 1987; Fung and Lau, 1988), but it is not clear whether enhanced sensitivity to nicotine's locomotor-activating effects is due to changes in receptor number inasmuch as none of these studies have carefully assessed the relationship between behavioral and

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biochemical changes using dose-response or time course analyses.

The observation that chronic nicotine treatment produces up-regulation of brain nicotine receptors coupled with the observation that tolerance develops to many of the effects of the drug led us (Marks *et al.*, 1983) to suggest that up-regulation occurs because of chronic agonist-induced desensitization or inactivation of the receptor. Similarly, Schwartz and Kellar (1985) have invoked the desensitization hypothesis to explain up-regulation in rat brain. Partial support for this notion comes from the observation that animals that have been chronically injected with nicotine show tolerance to nicotine-induced release of corticosterone (Benwell and Balfour, 1979; Balfour, 1980; Caggiula *et al.*, 1991), adrenocorticotropin (Sharp and Beyer, 1986) and prolactin (Sharp *et al.*, 1987; Hulihan-Giblin *et al.*, 1990). Rats that have been chronically injected with nicotine fail to exhibit nicotine-induced release of prolactin for as long as 8 days after chronic injection is stopped (Hulihan-Giblin *et al.*, 1990) which suggests that chronic nicotine injection may evoke a long-lasting decrease in nicotinic receptor function.

Studies with cell lines that have been exposed chronically to nicotinic agonists have shown that chronic treatment results in attenuated responses to nicotine. For example, PC12 cells that have been exposed chronically to nicotinic agonists exhibit decreases in agonist-induced uptake of Na^+ (Simasko *et al.*, 1986), and Rb^+ (Robinson and McGee, 1985) as well as efflux of Rb^+ (Lukas, 1991). Interestingly, several days were required before detectable recovery of function occurred after treatment was stopped.

Although cell lines can provide reliable data, it is possible that such studies do not provide adequate models for brain nicotinic receptor function, especially because the PC12 lines that were used in the studies described above do not contain the *alpha*-4 variant of the nicotinic receptor (Rogers *et al.*, 1992). Because more than 90% of brain nicotinic agonist binding occurs at receptors that contain the *alpha*-4 receptor isoform (Flores *et al.*, 1992), studies utilizing brain tissue are required. Two such studies have been reported, but the results obtained are conflicting. Chronic nicotine-treated rats showed decreased nicotinic agonist-induced release of acetylcholine from cortical and hippocampal slices (Lapchak *et al.*, 1989), but striatal synaptosomes obtained from rats that had been chronically treated with the nicotinic agonist, anatoxin, were more sensitive to nicotine-induced dopamine release (Rowell and Wonnacott, 1990). It is not clear whether these results differ because different agonists were used for the chronic treatment, because different brain regions were analyzed or because different assay procedures were used.

Recently, we have characterized two different assays that measure functional nicotinic responses in mouse brain: stimulation of [^{3}H]dopamine release from striatal synaptosomes (Grady *et al.*, 1992) and stimulation of $^{86}Rb^+$ efflux from synaptosomes of several mouse brain regions (Marks *et al.*, 1993). Several lines of indirect evidence suggest that these two processes may involve different types of nicotinic receptors. For example, neuronal bungarotoxin, which is a potent inhibitor of nicotinic receptors containing the *alpha*-3 subunit and a poor inhibitor of *alpha*-4-containing receptors (Bertrand *et al.*, 1990; Luetje *et al.*, 1990), is a potent inhibitor of nicotine-evoked dopamine release from striatum (Grady *et al.*, 1992) whereas this toxin is relatively ineffective in blocking nicotine-evoked efflux of $^{86}Rb^+$ from synaptosomes prepared from midbrain, primarily thalamic, regions (Marks *et al.*, 1993). Consequently, both of these assays were used to assess the effects of chronic nicotine infusion on brain nicotinic receptor function. The results indicate that tolerance develops to the effects of nicotine after chronic treatment when measured either behaviorally or biochemically, but that the magnitude of the biochemical tolerance depends upon the response measured and the tissue examined.

Materials and Methods

Materials. The following materials were obtained from Sigma Chemical Co. (St. Louis, MO): Tris, Tris hydrochloride, L-ascorbic acid, polyethylenimine, L-nicotine base. Percoll was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Sucrose and HEPES (hemisodium salt) were obtained from Boehringer-Mannheim (Indianapolis, IN). The scintillation fluid, BudgetSolve, was purchased from Research Products International (Mount Prospect, IL). The radioisotopes [7,8-^{3}H]dopamine (40–60 Ci/mmol) and [N-methyl-^{3}H] L-nicotine (75 Ci/mmole) were obtained from Amersham Corp. (Arlington Heights, IL) and carrier-free ^{86}RbCl was obtained from DuPont NEN (Boston, MA). All other chemicals were reagent quality.

Mice. Female mice of the C57BL/6J/lbg strain were between 60 and 90 days of age at the time of surgery. The mice were obtained from the breeding colony of the Institute for Behavioral Genetics, University of Colorado (Boulder, CO). Before surgery, five animals were housed per cage. Mice were allowed free access to food (Wayne Lab Blox) and water. The animal colony was maintained on a 12-hr light:12-hr dark cycle (lights on 7 A.M. to 7 P.M.).

Surgery. A cannula made of silastic tubing was implanted in the right jugular vein of each mouse using the method of Barr *et al.* (1979). Mice were anesthetized for surgery by injection of pentobarbital (50 mg/kg) and chloral hydrate (100 mg/kg). Three to five hours after a mouse awakened from the anesthesia, it was transferred to an individual treatment cage (15 × 15 × 30 cm; length by width by height) and its cannula was connected to polyethylene tubing attached to a glass syringe mounted on a Harvard Infusion Pump (Harvard Instruments, South Natick, MA). Continuous infusion with sterile saline at a rate of 35 μl/hr was then begun.

Chronic nicotine treatment. Drug treatment was started after a 2-day recovery period during which the mice were infused with saline. Saline infusion was continued for control mice, whereas one of five nicotine doses was used for the treated mice. The nicotine infusion rates used were 0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg/hr. Those mice being treated with one of the three lower doses were infused with that dose on the first drug treatment day and each treatment day thereafter. Those mice treated with either 2.0 or 4.0 mg/kg/hr were infused at one-half their final dose on the first day of treatment after which they were infused at their final treatment dose. Mice were infused with the final treatment dose for 10 days before they were tested for tolerance.

Measurement of acute responsiveness to nicotine (tolerance test). On the 10th day of drug treatment, each mouse was tested for its response after an acute intraperitoneal injection (0.01 ml/g) of saline and 1.0 mg/kg of nicotine. Two hours after cessation of treatment, saline was injected. Activity in the Y-maze and body temperature were then measured as described previously (Marks *et al.*, 1985a). Two hours after completion of this base-line measurement, 1.0 mg/kg of nicotine was administered and activity in the Y-maze and body temperature were again measured. The Y-maze test was conducted for 3 min beginning 5 min after injection of either saline or nicotine. Body temperature was measured 15 min after injections using a Thermalert rectal probe (Bailey Instruments, Saddlebrook, NJ). These times were chosen because earlier studies have demonstrated maximal effects of nicotine on these measures are seen at these test times (Marks *et al.*, 1985a). Results obtained from mice injected with both saline and nicotine did not differ from the results obtained from mice injected

with saline alone or nicotine alone. Double testing of the mice allowed the determination of both base-line responses and the effects of nicotine in each animal.

Upon completion of the tolerance tests, mice were returned to their infusion cages and chronic drug administration was continued.

Tissue preparation. Chronic drug treatment was discontinued 2 hr before each mouse was sacrificed by cervical dislocation. After sacrifice, the brain was removed from the skull, placed on an ice-cold platform and three brain areas [striatum, cerebral cortex and midbrain (thalamus and mesencephalon)] were dissected and placed in 10 volumes of ice-cold 0.32 M sucrose buffered at pH 7.5 with 5 mM HEPES. The tissue was homogenized by hand in a glass-Teflon tissue grinder using 16 strokes. The P2 fraction of each brain area was then prepared from the supernatant obtained after centrifugation for 10 min at 1000 × *g* followed by further centrifugation at 12,000 × *g* for 20 min. Inasmuch as mouse striata are small and little enhancement of signal intensity has been obtained after further purification of the P2 fraction, this P2 pellet was used as the source of tissue for the study of [^{3}H]dopamine release from striatum. The P2 pellets obtained from cortex and midbrain were further processed by centrifugation on Percoll gradients using a modification of the method of Nagy and Delgado-Escueta (1984) described previously (Grady *et al.*, 1992). The method uses a three-step discontinuous Percoll gradient (7.5, 10, 16%). After centrifugation for 20 min at 15,000 × *g*, the synaptosomes that formed a diffuse band above the 16% Percoll layer were harvested for use in the measurement of $^{86}Rb^+$ efflux. The addition of the Percoll gradient centrifugation increases the signal observed for nicotine-stimulated $^{86}Rb^+$ (Marks *et al.*, 1993).

[^{3}H]Nicotine binding. [^{3}H]Nicotine was purified by the method of Romm *et al.* (1990) before use. The binding assays were conducted on whole particulate fractions prepared from aliquots of P2 fractions for all three brain areas at 4°C as described previously (Marks *et al.*, 1986c). The final concentration of [^{3}H]nicotine was 21.4 ± 1.7 nM. Blanks were determined by including 10 μM unlabeled L-nicotine in the assay tubes. Incubation time was 2 hr.

Protein assay. Protein was assayed using the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

[^{3}H]Dopamine release. The release of [^{3}H]dopamine from striatal P2 preparations was measured as described previously (Grady *et al.*, 1992). Tissue was loaded by incubation with 0.1 μM [^{3}H]dopamine for 5 min at 37°C. After the incubation step, the tissue was collected by filtration onto 6-mm glass fiber filters. Each filter was transferred to a platform that allowed continuous superfusion of the sample. Buffer was applied to the filters by a peristaltic pump (Cassette Pump; Manostat, New York, NY) and drawn through the filters with a second peristaltic pump (Polystatic Pump, Buchler Instruments, Lexana, KS). The superfusion was conducted at room temperature (21 ± 1°C) using a flow rate of 0.2 to 0.3 ml/min. Buffer composition was (mM): NaCl, 128; KCl, 2.4; $CaCl_2$, 3.2; $MgSO_4$, 1.2; KH_2PO_4, 1.2; HEPES, 25; dextrose, 10; ascorbic acid, 1 mM pargyline, 10 μM, pH 7.5. After a 15-min wash period, samples were exposed to 10 μM nicotine for 1 min and 10 min later were exposed to 21.2 mM K^+ for 1 min. Exposure to the test solutions was achieved by simultaneously closing a valve supplying standard perfusion buffer while opening a valve supplying perfusion buffer containing the test solution (either nicotine or elevated K^+). One fraction was collected in 6-ml scintillation vials each minute. Radioactivity was determined after the addition of 3.5 ml of Budget-Solve Scintillation Cocktail with an LS 1800 Scintillation Counter (Beckman Instruments, Fullerton, CA). Counting efficiency was 45%.

$^{86}Rb^+$ efflux. The efflux of $^{86}Rb^+$ from synaptosomal fractions of cerebral cortex and midbrain prepared by Percoll gradient centrifugation was measured as described previously (Marks *et al.*, 1993). Tissue was loaded with $^{86}Rb^+$ by incubation for 45 min with 4 μCi $^{86}Rb^+$ at room temperature (21 ± 1°C) in an incubation volume of 35 μl. After the uptake period, tissue was collected by filtration on 6-mm glass fiber filters under gentle vacuum. Perfusion and stimulation by exposure to 10 μM L-nicotine were essentially the same as described above for the measurement of [^{3}H]dopamine release with the following exceptions: flow rate was 0.7 to 0.8 ml/min, wash time was 12 min before exposure to 10 μM L-nicotine for 1 min and no K^+ stimulation was conducted. The buffer used in the $^{86}Rb^+$ efflux experiments was: 134 mM NaCl, 1.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 20 mM HEPES, 20 mM dextrose, pH = 7.5. Two samples were collected in 12 × 75 mm culture tubes each minute. Radioactivity was determined using a Cobra Auto-Gamma Counter (Packard Instruments, Meriden, CT) at a counting efficiency of 25%.

Data calculations. The data for both [^{3}H]dopamine release and $^{86}Rb^+$ efflux were plotted as cpm in each fraction *vs.* time elapsed. The peaks were identified and the peak size was determined by summing the radioactivity released above base line. Basal release was determined by averaging the cpm in the fractions immediately preceding and after the peak. Peak size for both types of experiments were normalized to correct for differences in amount of tissue or levels of radioactivity. The [^{3}H]dopamine release data were normalized by dividing the amount of [^{3}H]dopamine release above the base line by the average base line underlying the peak, whereas the $^{86}Rb^+$ efflux data were normalized by dividing the amount of $^{86}Rb^+$ efflux above the base line by the amount of $^{86}Rb^+$ remaining in the tissue.

An overall behavioral response index was calculated from the results obtained for Y-maze crosses, Y-maze rears and body temperature tests to provide a general indication of the degree of tolerance development. The responses observed for these three tests after an acute injection of nicotine have been observed to be closely related to each other either in response to chronic nicotine treatment (Marks *et al.*, 1986b, 1991) or when measured in various inbred mouse strains (Marks *et al.*, 1989). The overall response index was calculated as follows: overall score = {(Y-maze crosses after nicotine/control Y-maze crosses) + (Y-maze rears after nicotine/control Y-maze rears) + [4°C/(control body temperature − body temperature after nicotine)]}/3. The score ranges from about 0 for maximum drug effect (no activity and a 4°C temperature decrease) to about 1.0 for no drug effect.

Both the EC_{50} and maximal response were calculated from results of the concentration-effect curves. These values were estimated by non-linear curve fitting of the untransformed data and by linear regression analysis of Eadie-Hofstee transformations of the data.

The effects of chronic drug treatment dose on behavioral responses, [^{3}H]nicotine binding, nicotine-stimulated and K^+-stimulated [^{3}H]dopamine release, nicotine-stimulated $^{86}Rb^+$ efflux and tissue $^{86}Rb^+$ content were analyzed by one-way analysis of variance followed by Duncan's New Multiple Range post-hoc test using the SPSS/PC statistical package.

Results

***In vivo* tolerance tests.** The acute responsiveness of the mice to nicotine was measured to determine the extent of behavioral tolerance after chronic nicotine infusion. The results of these tests are presented in figure 1. Chronic nicotine infusion did not change the response to saline injection for the Y-maze crosses, Y-maze rears and body temperature measures. In contrast, magnitude of the effect observed after injection of 1.0 mg/kg of nicotine decreased as infusion dose increased for all three individual measures. The degree of tolerance was not identical for each response. Although both Y-maze crosses and hypothermia were virtually unaffected by an acute injection of 1.0 mg/kg in those mice treated with the highest infusion dose of nicotine, this acute drug dose still depressed the number of Y-maze rears. Tolerance to the effects of the challenge dose of nicotine on body temperature was noted after infusion with 0.5 mg/kg/hr of nicotine, but statistically significant tolerance for the two Y-maze measures was not observed for chronic doses below 2.0 mg/kg/hr. The pattern observed for overall responsiveness to nicotine serves to summarize the pattern of tolerance development.

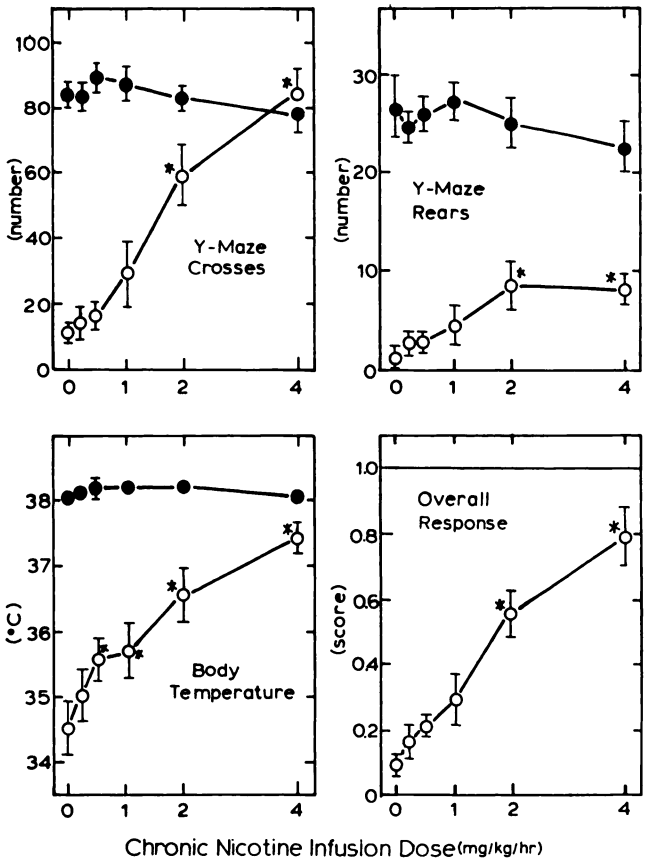

Fig. 1. Behavioral and physiological responses of C57BL/6 mice after chronic nicotine infusion. C57BL/6 mice that had been chronically infused with the indicated doses of nicotine were tested for several responses after an injection of saline (open symbols) or 1.0 mg/kg of nicotine (closed symbols). Values represent mean ± S.E. for 8 to 10 mice per group. Results of the one-way ANOVA after saline injection were: Y-maze crosses, $F(5,54) = 0.48$; Y-maze rears, $F(5,54) = 0.58$ body temperature, $F(5,54) = 0.53$ (no significant effects of nicotine treatment). Results of one-way ANOVA after 1.0 mg/kg of nicotine: Y-maze crosses, $F(5,54) = 15.69$ ($P < .001$); Y-maze rears, $F(5,54) = 4.01$ ($P < .01$); body temperature, $F(5,54) = 8.53$ ($P < .001$) and overall response, $F(5,54) = 15.91$ ($P < .001$). Those values differing significantly from the responses of saline-infused mice after acute nicotine injection are indicated by asterisks (*).

[^{3}H]Nicotine binding. The effects of chronic nicotine infusion on the binding of [^{3}H]nicotine was determined in particulate preparations isolated from P2 fractions of cerebral cortex, midbrain and striatum. The binding was determined using a single concentration of [^{3}H]nicotine (21.7 nM) that measures near-maximal number of binding sites. The results of the binding experiments are shown in figure 2. Chronic nicotine infusion resulted in significant increases in [^{3}H]nicotine binding in both cortex and midbrain. Maximal increases were observed after infusion with the relatively low dose of 0.5 mg/kg/hr. Chronic drug treatment resulted in an increase of approximately 60% in cortex and an increase of approximately 40% in midbrain. The number of binding sites also tended to increase in striatum, but these increases were not significant.

[^{3}H]Dopamine release. Chronic nicotine infusion resulted in a dose-dependent decrease in nicotine-stimulated (10 μM) [^{3}H]dopamine release (fig. 3). The release from striatal P2 fractions prepared from mice infused with both 2.0 and 4.0 mg/kg/hr of nicotine were significantly lower than the release measured from control tissue. Nicotine-stimulated [^{3}H]dopamine release was approximately 20% lower in mice treated with 4.0 mg/kg/hr nicotine than it was in control mice. In contrast, K^+-stimulated [^{3}H]dopamine release was unaffected by chronic nicotine infusion as illustrated in the inset to figure 3.

In order to determine whether the effect of chronic nicotine administration on nicotine-stimulated [^{3}H]dopamine release arose from a change in the maximum rate of release and/or the EC_{50} for L-nicotine-stimulated [^{3}H]dopamine release, stimulation was measured after exposure to buffer or one of five nicotine concentrations in tissue prepared from control mice and mice infused with 4.0 mg/kg/hr. The results of these experiments are shown in figure 4. Chronic drug treatment had no effect on the EC_{50} value, but the chronic treatment did cause a decrease in the maximal nicotine-stimulated [^{3}H]dopamine release. The inset to figure 4, in which the results are presented as Eadie-Hofstee plots, further illustrates that the chronic treatment decreased the maximal nicotine-stimulated [^{3}H]dopamine release without effect on the EC_{50} for L-nicotine.

$^{86}Rb^+$ efflux. The results presented in figure 5 summarize the effects of chronic nicotine treatment on the $^{86}Rb^+$ efflux stimulated by a 1-min exposure to 10 μM L-nicotine. Although chronic drug treatment had no significant effect on nicotine-stimulated $^{86}Rb^+$ efflux from cortical synaptosomes, the chronic nicotine treatment resulted in a significant decrease in nicotine-stimulated $^{86}Rb^+$ efflux from midbrain synaptosomes. The magnitude of the nicotine-stimulated $^{86}Rb^+$ efflux from tissue prepared from mice treated with every dose of nicotine was significantly lower than that of controls. The nicotine-stimulated $^{86}Rb^+$ efflux observed for tissue from mice treated with 4.0 mg/kg/hr of nicotine was approximately one-half that measured for saline-infused animals.

The effect of chronic nicotine infusion on the total amount of tissue $^{86}Rb^+$ was analyzed to determine whether chronic drug treatment affected tissue uptake of the ion. The results of this experiment are summarized in the insets to figure 5. Chronic drug infusion had no effect on total $^{86}Rb^+$ uptake.

In order to determine whether the effect of chronic nicotine administration on nicotine-stimulated $^{86}Rb^+$ efflux arose from a change in the maximum rate of release and/or the EC_{50} for L-nicotine, $^{86}Rb^+$ efflux after stimulation with buffer or one of five nicotine concentrations was measured in cortical and midbrain synaptosomes prepared from control mice and mice infused with 4.0 mg/kg/hr. The results of these experiments are summarized in figure 6. The shape of the concentration-response curves for $^{86}Rb^+$ efflux from cortical synaptosomes determined for control and treated mice were very similar. No significant difference in either maximal response or EC_{50} value for L-nicotine was observed. The presentation of these results as Eadie-Hofstee plots also illustrates that chronic drug treatment had no significant effect on cortical nicotine-stimulated $^{86}Rb^+$ efflux (inset to left panel of fig. 6).

The concentration-response curves constructed for nicotine-stimulated $^{86}Rb^+$ efflux from synaptosomes prepared from midbrains of control and treated mice differ substantially. Although no difference in the EC_{50} values was observed, a significant reduction in response was noted for the tissue prepared from drug-treated mice after stimulation with each of the five concentrations of L-nicotine. The inset to the right panel of figure 6, in which the results are presented as Eadie-Hofstee plots, further illustrates that the chronic treatment decreased the maximal nicotine-stimulated $^{86}Rb^+$ efflux without effect on the EC_{50} for L-nicotine.

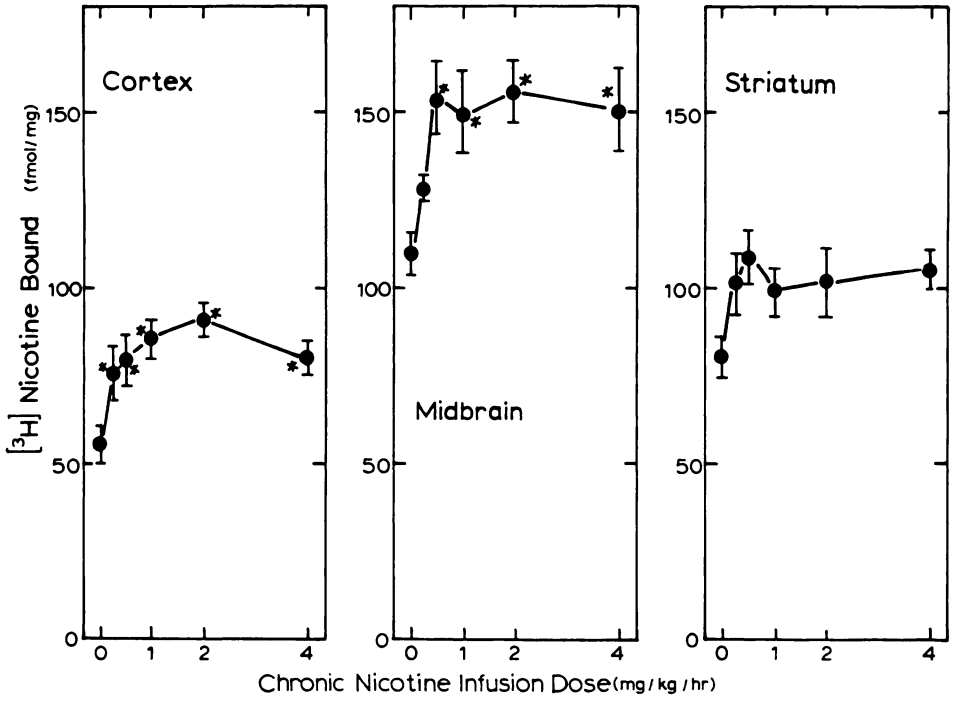

Fig. 2. [^{3}H]Nicotine binding after chronic nicotine infusion. Whole particulate material prepared from P2 fractions of cerebral cortex, midbrain and striatum was assayed for the binding of [^{3}H]nicotine (21.4 ± 1.7 nM). Symbols represent mean ± S.E. of specific [^{3}H]nicotine binding for 8 to 10 mice per group. The one-way ANOVA indicated that chronic nicotine treatment significantly changed [^{3}H]nicotine binding in cerebral cortex [$F(5,51) = 3.92$; $P < .01$] and midbrain [$F(5,54) = 3.90$; $P < .01$], but not striatum [$F(5,58) = 1.82$; $P > .05$]. Those values differing significantly from control binding are designated with asterisks (*).

Discussion

Mice chronically treated with nicotine developed tolerance to the effects of nicotine *in vivo* and also showed increases in the number of binding sites measured with [^{3}H]nicotine. These results are consistent with previous reports in which tolerance development and ligand binding were measured after chronic nicotine treatment (Marks *et al.*, 1983, 1985b, 1986a,b, 1991). The *in vivo* responsiveness decreased approximately linearly with increase in infusion dose, whereas binding site densities in the three brain areas measured appeared to be hyperbolic functions of treatment dose and showed maximal increases after treatment with the relatively low dose of 0.5 mg/kg/hr. An apparent uncoupling of tolerance from binding site density after chronic treatment has been noted previously (Marks *et al.*, 1991; Collins *et al.*, 1990).

The pattern emerging from the measurements of receptor function is somewhat complex. Tolerance development after chronic drug treatment was observed for nicotine-stimulated [^{3}H]dopamine release from striatum and $^{86}Rb^+$ efflux from midbrain, but not for $^{86}Rb^+$ efflux from cortex. For the two responses that were affected by chronic treatment, the magnitude of both of these responses decreased with an increase in chronic nicotine infusion dose. However the pattern of response differed between the two functions. The [^{3}H]dopamine release decreased gradually with treatment dose, whereas the $^{86}Rb^+$ efflux decreased as the treatment dose increased in a pattern resembling a rectangular hyperbola. The loss in responsiveness did not arise from increases in the EC_{50} values for activation by nicotine, but from a lower maximal response.

One possible explanation for the diminished functional responses observed in this study is that residual nicotine remained in the tissue after the cessation of treatment and that this residual drug desensitized receptor responses. This explanation is unlikely for several reasons. Although residual tissue nicotine was not measured, metabolism of nicotine in the mouse is very rapid (Hatchell and Collins, 1980; Petersen *et al.*, 1984) such that the 2-hr withdrawal period that occurred between the cessation of treatment and the beginning of the tissue preparation represents more than 10 half-lives and, therefore, should have been adequate to reduce substantially the levels of nicotine in the animal. In addition the tissue preparation involved several washing steps followed by a 10- to 15-min washout period after the tissue was prepared and loaded with isotope before exposure to nicotine. The wash steps would serve to remove additional residual nicotine. Finally, we have begun studies of the time courses for the recovery of nicotinic receptor function after the cessation of treatment. The results of these experiments indicate that both [^{3}H]dopamine release and $^{86}Rb^+$ efflux remain depressed for several days after the cessation of chronic drug treatment (unpublished observations). It therefore seems likely that the reduced function reported in the present study does not arise from simple receptor desensitization caused by the presence of residual tissue nicotine.

The diminished response observed for these two measures is consistent with the results of previous studies in which reduced nicotinic function was observed after chronic nicotinic agonist exposure as measured by hormonal responses *in vivo* (Benwell and Balfour, 1979; Balfour, 1980; Sharp and Beyer, 1986; Sharp *et al.*, 1987; Hulihan-Giblin *et al.*, 1990), by acetylcholine release from hippocampal and cortical slices *in vitro* (Lapchak *et al.*, 1989) and by ion flux in cultured cells (Robinson and McGee, 1985; Simasko *et al.*, 1986; Lukas, 1991). However, the magnitude of the changes observed in mouse brain (20% decrease in response for [^{3}H]dopamine release, 50% decrease for $^{86}Rb^+$ efflux) are in general lower than those reported for hormonal response and acetylcholine release in rats, and ion flux in cultured cells. Whether the smaller change in response observed for the mice represents species differences in responsiveness to chronic nicotine or establishes a maximum change compatible with survival is not established.

In contrast to the decreased responses observed in striatum (20%) and midbrain (50%), chronic nicotine infusion had no significant effect on nicotine-stimulated $^{86}Rb^+$ release in cortex.

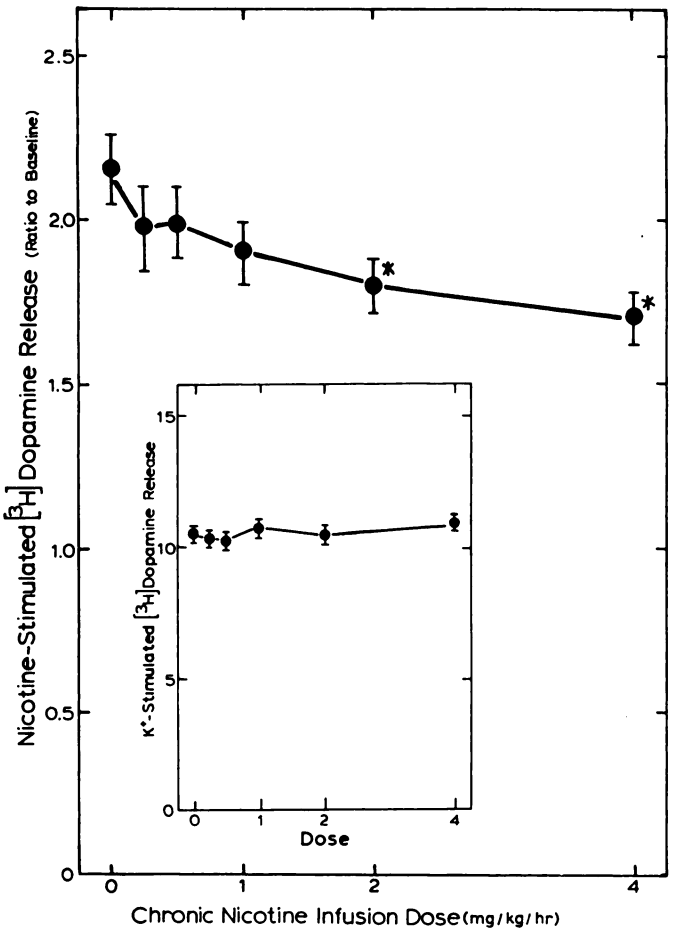

Fig. 3. Nicotine-stimulated [^{3}H]dopamine release from striatal P2 fractions after chronic nicotine infusion. Striatal P2 fractions were prepared, loaded with [^{3}H]dopamine and stimulated by exposure to 10 μM nicotine (main panel) or 21.2 mM K^+ (inset) for 1 min. Results represent mean ± S.E. for 10 to 14 individual experiments. Chronic nicotine infusion significantly reduced nicotine-stimulated [^{3}H]dopamine release [$F(5,79) = 2.93$; $P < .05$] but had no measurable effect on K^+-stimulated [^{3}H]dopamine release [$F(5,78) = 0.74$; $P > .05$]. Values differing from the response of saline-infused mice are indicated by asterisks (*).

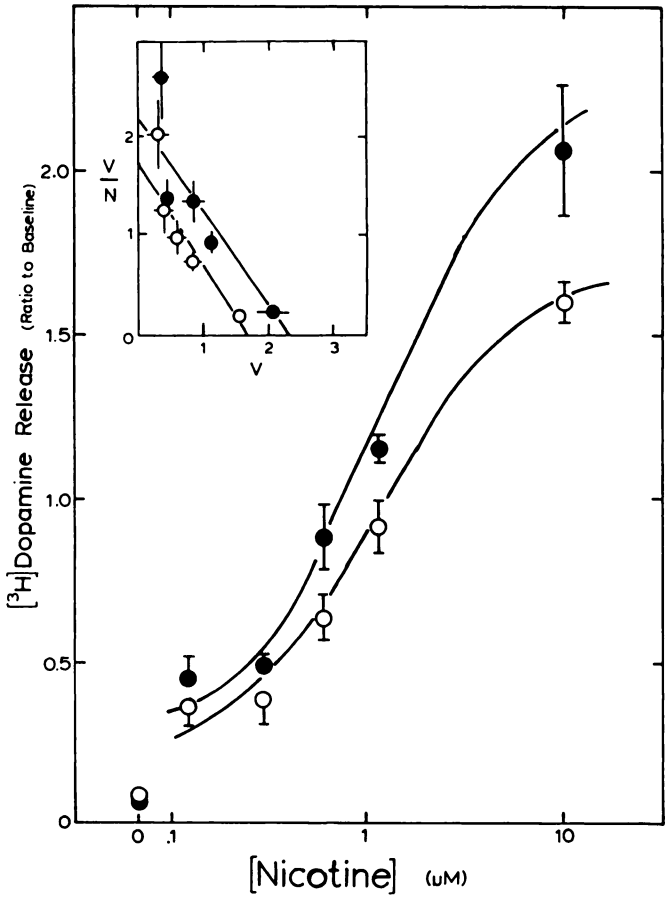

Fig. 4. Concentration-response curves for nicotine stimulation of [^{3}H] dopamine release. Striatal P2 fractions were prepared from control mice (closed symbols) and mice treated with 4.0 mg/kg/hr of nicotine (open circles), loaded with [^{3}H]dopamine and stimulated by exposure for 1 min to the indicated concentrations of nicotine. Points represent mean ± S.E. of data from six separate experiments. Curves in the main panel are log concentration-effect curves, whereas those in the inset are Eadie-Hofstee plots of the same data. The EC_{50} values calculated for the control (0.80 ± .20 μM) and nicotine-treated groups (0.71 ± .16 μM) did not differ, but the maximum nicotine-stimulated [^{3}H]dopamine release was higher in control (1.98 ± .42) than in treated (1.48 ± .26) mice.

The reasons for this differential response to chronic nicotine treatment are not clear, but several possible explanations may be advanced. 1) Different receptor subtypes may mediate the responses to nicotine stimulation in different brain regions. Molecular cloning studies have revealed that the nicotinic receptor family consists of several agonist recognition subunits (*alpha*) and several structural (*beta*) subunits (see Deneris *et al.*, 1991 for review). The mRNA encoding these subunits are differentially distributed in rat (Deneris *et al.*, 1989; Boulter *et al.*, 1990; Goldman *et al.*, 1987; Wada *et al.*, 1989; Seguela *et al.*, 1993) and mouse (Marks *et al.*, 1992) brain. Therefore, the existence of different receptor subtypes in different brain areas, and even within a brain area, is a distinct possibility. The physiological and pharmacological properties of receptor subtypes when expressed in *Xenopus* oocytes differ in their sensitivity to agonists (Luejte and Patrick, 1991) and antagonists (Luetje *et al.*, 1990; Bertrand *et al.*, 1990), as well as the relative rate of desensitization (Gross *et al.*, 1991; Couturier *et al.*, 1990). Inasmuch as the substantia nigra, which projects to the caudate-putamen (striatum) expresses mRNA for many receptor subtypes (Wada *et al.*, 1989; Deneris *et al.*, 1989; Marks *et al.*, 1992), the possibility exists that nicotinic receptor-mediated dopamine release may be regulated by any one of several nicotinic receptors. Such potential receptor heterogeneity may explain the relatively poor correlations between the potency of agonists as inhibitors of ligand binding and as stimulators of dopamine release in striatum (Grady *et al.*, 1992) in contrast to the relatively good correlations obtained for $^{86}Rb^+$ efflux in midbrain (Marks *et al.*, 1993), in which the mRNA for relatively few nicotinic receptor subunits is found. Although the different responses observed for nicotine-stimulated $^{86}Rb^+$ efflux in cortex and midbrain might arise because of subtype differences, this possibility seems less likely for $^{86}Rb^+$ efflux than for [^{3}H] dopamine release. 2) The receptor subtypes present in the two brain areas do not differ, but the mechanisms by which the receptors are processed in these brain areas may differ. Although many subtypes of nicotinic receptors have been identified, mRNA for only a few are widely expressed in the brain (particularly the subunits designated *alpha*-4, *alpha*-7, and *beta*-2) (Wada *et al.*, 1989; Seguela *et al.*, 1993). Therefore, if mRNA levels reflect likely receptor levels, perhaps only a few receptor subtypes are quantitatively important in most parts of the central nervous system. For example, immunological experi-

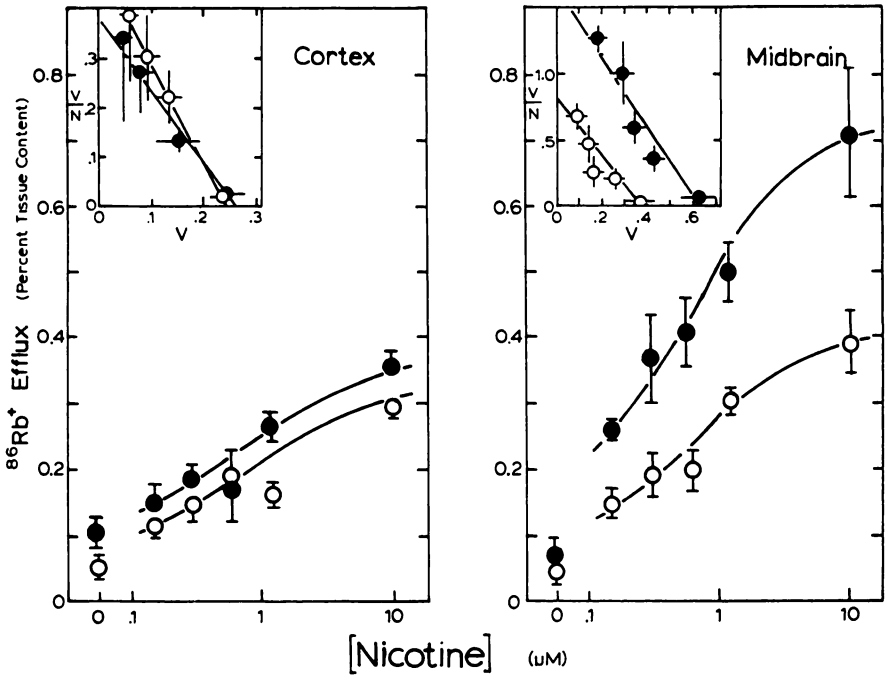

Fig. 5. Nicotine-stimulated $^{86}Rb^+$ efflux from cerebral cortical and midbrain synaptosomes. Synaptosomes prepared from cerebral cortex and midbrain of mice treated with the indicated doses of nicotine were prepared using Percoll gradients, loaded with $^{86}Rb^+$ and subsequently stimulated by exposure to 10 μM nicotine for 1 min. Points in the main panels are mean ± S.E. of 10 to 12 individual experiments for nicotine-stimulated $^{86}Rb^+$ efflux and points in the insets mean ± S.E. for 10 to 12 individual experiments for tissue $^{86}Rb^+$ content after nicotine stimulation. Chronic nicotine infusion had no significant effect on nicotine-stimulated $^{86}Rb^+$ efflux from cerebral cortical synaptosomes [$F(5,63) = 0.50$; $P > .05$] but treatment significantly reduced nicotine-stimulated $^{86}Rb^+$ release from midbrain synaptosomes [$F(5,64) = 6.69$; $P < .001$] . Chronic drug treatment had no effect on tissue $^{86}Rb^+$ content in either cortex [$F(5,64) = 0.63$; $P > .05$] or midbrain [$F(5,64) = 0.62$; $P > .05$]. Values significantly different from corresponding values for saline-infused mice are indicated by asterisks (*).

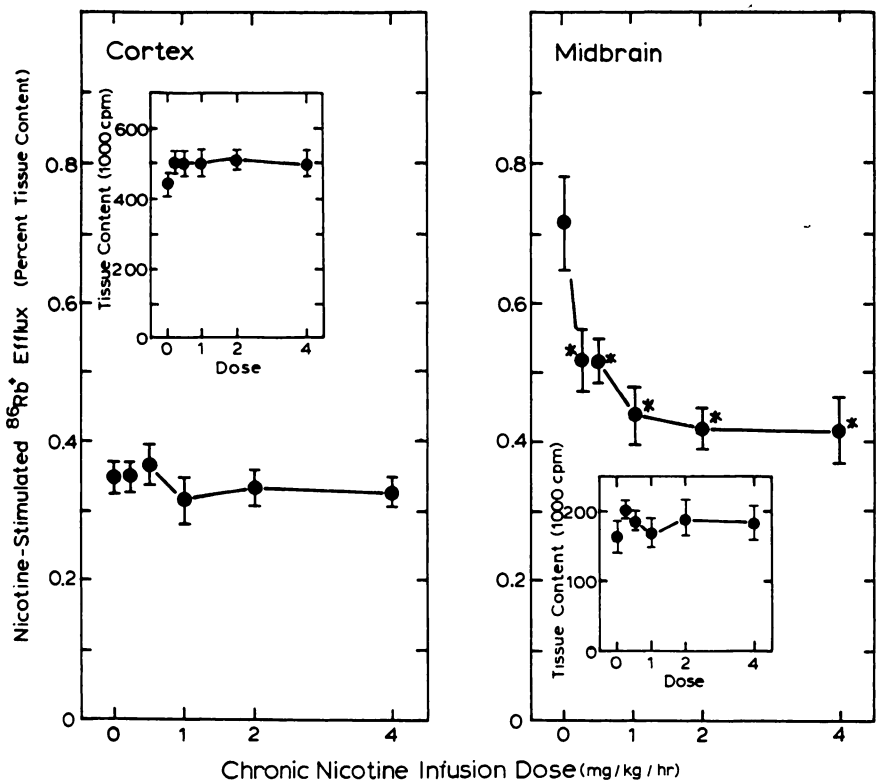

Fig. 6. Concentration-response curves for nicotine stimulation of $^{86}Rb^+$ efflux from cortex and midbrain synaptosomes. Synaptosomes were prepared by Percoll gradient centrifugation from cortex and midbrain of control mice (closed symbols) and mice treated with 4.0 mg/kg/hr of nicotine (open circles). The synaptosomes were subsequently loaded with $^{86}Rb^+$ and stimulated by exposure to the indicated concentrations of nicotine for 1 min. The points represent mean ± S.E. of eight separate experiments. Curves in the main panels are the concentration-effect curves, whereas curves in the insets are Eadie-Hofstee plots of the same data. Chronic nicotine infusion had no significant effect on the EC_{50} for nicotine in either cortex (0.68 ± 0.31 μM for control; 0.40 ± .23 μM for treated) or midbrain (0.33 ± .10 μM for control; 0.40 ± .15 μM for treated) tissue. Chronic drug treatment did not significantly change the maximum response in cortex (0.24 ± .06 for controls; 0.21 ± .04 for treated mice) but chronic treatment did reduce the response in midbrain (0.60 ± .05 for controls; 0.33 ± .05 for treated mice).

ments (Whiting and Lindstrom, 1986; Whiting *et al.*, 1987; Lindstrom *et al.*, 1990; Flores *et al.*, 1992) suggest that more than 90% of high-affinity nicotine binding sites correspond to a single receptor subtype (*alpha*-4 to *beta*-2). Analysis of the proposed structure of the receptor subunits indicates that the receptors contain extracellular sites for glycosylation as well as intracellular phosphorylation sites and proteolytic sites (Deneris *et al.*, 1991). Differences among brain areas in the extent of enzymatic modification of the receptors could explain different functional responses after chronic drug treatment. 3) The receptor subtypes are similar, but the localization of the receptors on cells within these areas differs subsequently allowing for or leading to different responses to chronic nicotine exposure. Even though a Percoll gradient centrifugation method was used to prepare synaptosomes from both cortex and midbrain, the organelles prepared by this method are not homogeneous (Nagy and Delgado-Escuada, 1984) and may differ somewhat among brain regions. Even if the general population of organelles isolated from different brain regions is similar, the function of nicotinic receptors in these two brain areas may be different (for example regulation of the release of different neurotransmitters). Quantitative autoradiographic analysis of

both [^{3}H]nicotine and α-[^{125}I]bungarotoxin binding after chronic nicotine treatment indicates that this treatment differentially affects the levels of binding sites in different brain nuclei (Pauly *et al.*, 1991). This result suggests that even though a major or single receptor subtype accounts for the binding measured with [^{3}H]nicotine, the response of the binding sites to chronic nicotine treatment varies with anatomical location. Functional role or cellular location could affect the manner in which a receptor is processed when it is chronically exposed to nicotine. 4) The assertion that chronic nicotine treatment evoked different changes in receptor function in different brain regions may be an artifact of calculation of the results of these experiments. Specifically, the results were calculated in terms of activity per unit of tissue. However, we have shown previously in brain tissue obtained from drug-naive mice that greater numbers of nicotine binding sites are correlated with enhanced $^{86}Rb^+$ efflux (Marks *et al.*, 1993). The change in the density of [^{3}H]nicotine binding sites and functional responses observed with treatment dose in striatum and midbrain appear to be mirror images of each other (binding increases, function decreases with similar effect of treatment dose). If the amount of efflux or release is calculated per high-affinity [^{3}H]nicotine binding site a dramatic reduction (approximately 45%) of [^{3}H] dopamine release and a 58% reduction in $^{86}Rb^+$ flux in midbrain, and 40% reduction in ion flux from cortex is estimated. If this calculation is a valid indicator of relative receptor function, chronic treatment results in an uncoupling of receptor number from function in all three brain areas. An uncoupling of receptor number and functional status was noted by Lapchak *et al.* (1989) for nicotine-induced acetylcholine release from brain tissue and by Lukas (1991) for $^{86}Rb^+$ efflux from clonal cell lines after chronic treatment with nicotinic agonists. Lukas (1991) proposed that because during chronic treatment agonist is continuously present, any attempted cellular response to compensate for the persistent receptor blockade would be futile inasmuch as the additional receptors would be exposed to agonist and be rendered nonfunctional. Such an explanation would also be applicable to animals chronically exposed to nicotine *in vivo*.

Whether any, or all, of these explanations applies to the differential responses observed in cortex and midbrain after chronic nicotine treatment remains to be determined.

The measures of functional tolerance and behavioral tolerance described in this study were not directly related in that receptor activity decreased as a hyperbolic function of nicotine dose, whereas behavioral sensitivity to nicotine decreased as a linear function of nicotine dose. It would appear that tolerance to the effects of nicotine *in vivo* cannot be fully explained by either the changes in the number of binding sites or in the functional status of the receptors as measured by [^{3}H]dopamine release or $^{86}Rb^+$ efflux. Clearly changes in addition to those measured by receptor number and function contribute to altered responsiveness *in vivo*.

In summary, the results reported here indicate that chronic nicotine treatment evokes behavioral tolerance to the effects of nicotine as well as increases in receptor binding that occur together with a decrease in function in most, if not all, brain regions. The mechanisms that underlie this functional downregulation are unknown, but such a change is consistent with tolerance to nicotine's effects on various behavioral and physiological processes.

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