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Research report

A single (−)-nicotine injection causes change with a time delay in the affinity of striatal D_2 receptors for antagonist, but not for agonist, nor in the D_2 receptor mRNA levels in the rat substantia nigra

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Abstract

The in vitro and in vivo effects of (−)-nicotine on dopamine D_2 receptors in the rat neostriatum have been studied using biochemical binding, in situ hybridization and immunocytochemistry. A single i.p. injection (1 mg/kg) of (−)-nicotine resulted in a reduction of the K_D value of the D_2 antagonist [^{3}H]raclopride binding sites in rat neostriatal membrane preparations at 12 h without any significant change in the B_{max} value. This action of (−)-nicotine was counteracted by pretreatment 15 min earlier with the nicotine antagonist mecamylamine (1 mg/kg, i.p.). However, the K_D and the B_{max} values of the D_2 agonist [^{3}H]NPA binding sites in the rat neostriatal membrane preparations were not significantly affected 0.5–48 h after a single i.p. injection with 1 mg/kg of (−)-nicotine. No significant change in neostriatal D_2 receptor mRNA levels was observed at any time interval after the (−)-nicotine injection. No significant change was observed in tyrosine hydroxylase (TH) immunoreactivity in either the substantia nigra or the neostriatum, nor in nigral TH mRNA levels during the time interval studied (4–24 h posttreatment). Furthermore, addition of low (10 nM) or high (1 μM) concentrations of (−)-nicotine in vitro to rat neostriatal membranes did not alter the characteristics of [^{3}H]raclopride or [^{3}H]NPA binding. These results indicate that a single (−)-nicotine injection can produce a selective and delayed increase in the affinity of D_2 receptors for the antagonist, but not for the agonist without modifying the levels of D_2 receptor mRNA, probably via the activation of central nicotinic receptors.

Keywords: Nicotine; Dopamine D_2 receptor; Receptor–receptor interaction; Tyrosine hydroxylase; In situ hybridization

1. Introduction

Early work on acute or chronic-intermittent systemic injection of (−)-nicotine provides evidence for (−)nicotine produced increases in dopamine (DA) turnover in a large number of DAergic systems, including mesolimbic and nigrostriatal DA neurones[4,5,14,15,16]. In addition, after intraventricular administration, single injection of (−)-nicotine led to dose-related increases of DA turnover within various parts of the neostriatum, the accumbens and the olfactory tubercle[4], indicating that central nicotinic receptors are involved in controlling DA release. The early in vitro work with (−)-nicotine only demonstrated enhancement of DA release in neostriatal tissue by high concentrations of (−)-nicotine[56]. Subsequently, it was demonstrated that (−)-nicotine in low concentrations can cause release of DA from neostriatum in a calcium-dependent manner[21,44,59] as well as inhibit DA uptake[28]. Recent work has implied that (−)-nicotine acts preferentially on mesolimbic DA neurones[8,18,27,48]. More specifically, the bursting activity of mesolimbic DA neurones appears to be increased by (−)-nicotine[22,39]. Quantitative receptor autoradiography in combination with 6-hydroxy-dopamine caused lesions of the ascending DA pathways indicates the existence of high affinity nicotinic cholinergic receptors on DA cell bodies and nerve terminals of mesolimbic and nigrostriatal DA neurones[11,25,50]. The major population of nicotinic receptors involved in control of DA release in the neostriatum following acute systemic injection with (−)-nicotine appears to

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be located on nigral DA cell bodies in animals in which cholinergic stimulation of substantia nigra has been performed in intact and peduncular pontine tegmental nucleus lesioned rats[6,7,57,59].

Following chronic (−)-nicotine treatment adaptive changes have been observed in DA turnover depending on the type of treatment performed (repeated daily injections or chronic infusion of nicotine via osmotic minipumps). Constant infusion of (−)-nicotine leads to reduction of DA utilization within the neostriatum[12,19,30]. Continuous infusion has been reported by some authors to increase the sensitivity of postsynaptic neostriatal DA receptors[18,19], while others have failed to see any changes in DA receptor binding parameters in the neostriatum after such treatments[31]. Still, others have reported reductions of D_2 agonist binding[29]. In addition, there is a lack of knowledge on how acute single (−)-nicotine injection may lead to alterations in DA receptor binding characteristics within the basal ganglia.

In order to clarify the acute effects of (−)-nicotine on neostriatal DA receptors, we have performed a time-course of the action of a single dose of (−)-nicotine on D_2 receptor binding characteristics, as well as on D_2 receptor mRNA levels in the neostriatum of male rats. Biochemical binding techniques involving both D_2 agonist and antagonist radioligands have been used. The dose of (−)-nicotine used was equivalent to that which results in a blood level of (−)-nicotine similar to that found in smokers[49]. As a presynapic correlate to the studies on neostriatal D_2 receptors, D_2 receptor mRNA levels, tyrosine hydroxylase (TH) mRNA levels and TH immunoreactivity (IR) have been analyzed in the ventral mesencephalon and TH immunoreactive nerve terminals in the striatum, using in situ hybridization and immunocytochemistry.

2. Materials and methods

2.1. Animal treatment and materials

Male, specific pathogen-free, adult Sprague–Dawley rats (body weight 200 g, B and K Universal, Stockholm, Sweden) were used. The animals were kept under regular lighting conditions (light on at 08.00 h and off at 20.00 h) and had free access to food pellets and tap water. The rats used in the in vivo studies were injected intraperitoneally (i.p.) with (−)-nicotine (1 mg/kg) or saline only (1 ml), respectively, at various time intervals before decapitation. In some experiments the nicotine receptor antagonist mecamylamine was given (1 mg/kg, i.p.) 15 min prior to the (−)-nicotine injection and the rats were sacrificed at the 12 h time-interval.

(−)-nicotine (−)-nicotine hydrogen(+)-tartrate; BDH Chemical, Poole, UK] was dissolved in saline. DA (Sigma) and raclopride (Astra Läkemedel, Södertälje, Sweden) were dissolved in a few drops of distilled water and diluted with incubation buffer on each experimental day. [^{3}H]Raclopride (specific activity 65–82 Ci/mmol) and [^{3}H]NPA (propylnorapomorphine, (−), [*N*-propyl-^{3}H(N)]-, specific activity 53–65 Ci/mmol) were purchased from New England Nuclear, Boston, MA, USA. All other chemicals used were of analytical grade and purchased from Sigma Chemical (USA) or Merck (USA).

2.2. Biochemical binding experiments

2.2.1. Membrane preparations from rat neostriatum

The procedure used to obtain the crude membrane preparations has been described elsewhere[35]. Briefly, the rats were decapitated with a guillotine at several times (0.5, 4, 12, 24 and 48 h) after i.p. injection with 1 mg/kg of (−)-nicotine. Brains were rapidly removed and placed on ice. Each brain was cut at the middle and the neostriata were removed with sharp forceps. The tissue was weighed, placed into a 50 ml polypropylene vial and sonicated twice for 15 sec each time (Soniprep 150, MSE, Crawley, UK) in 5 ml of ice-cold 50 mM Tris buffer (pH 7.4), containing 5 mM $MgCl_2$, 1 mM EDTA and 0.01% L-(+)-ascorbic acid. The homogenate (about 12 mg of tissue wet weight/ml) was centrifuged at 45,000 *g* for 10 min at 4° C (Sorvall RC-5B, DuPont Instruments, USA). The membrane pellet was resuspended by sonication and preincubated in 20 vols. of Tris buffer for 30 min at 37° C to remove endogenous ligands. The preincubation was stopped by adding the same volume of ice-cold Tris buffer followed by another centrifugation. The resulting pellet was resuspended in 5 ml of Tris buffer and used immediately.

2.2.2. Saturation experiments with the D_2 antagonist [^{3}H]raclopride or D_2 agonist [^{3}H]NPA

In order to study the in vivo effects of (−)-nicotine on D_2 receptors, saturation curves with ten concentrations (1–35 nM) of the DA D_2 antagonist [^{3}H]raclopride[32] or ten concentrations (0.1–5.0 nM) of the D_2 agonist [^{3}H]NPA[12] were obtained under equilibrium conditions by incubating the neostriatal membranes obtained from the (−)-nicotine-treated rats (final incubation volume was 0.5 ml) in 50 mM Tris buffer for 30 min at 25° C. The incubation was terminated by using a BRANDEL M-24 cell harvester (Gaithersburg, MD, USA) through GF/B glass fibre filters (Whatman International, Maidstone, UK). The filters were washed rapidly (< 8 sec) with 3 × 5 ml of ice-cold Tris buffer. The radioactivity content of the filters was counted in 5 ml of scintillation cocktail

FLO-SCINT V (Packard, Groningen, The Netherlands) by liquid scintillation spectrometry (Beckman LS 1800, Irvine, CA, USA; efficiency 49–50%). Non-specific binding was defined as the binding in the presence of 100 μM of DA for the [^{3}H]raclopride experiments and 1 μM of the D_2 antagonist raclopride for the [^{3}H]NPA experiments. The filter binding was less than 100 dpm. Specific tissue binding, expressed as fmol/mg protein, was calculated by subtracting the non-specific binding from the total binding and amounted to 80–90% of the total binding in all experiments. Protein content was determined using bovine serum albumin (BSA) as a standard[37]. The final concentration of the membranes was about 0.1 mg protein/ml of incubation medium.

In order to study the in vitro effects of (-)-nicotine on D_2 receptors, similar experiments were performed with [^{3}H]raclopride or with [^{3}H]NPA by incubating the neostriatal membranes, obtained from normal rat brains and prepared in the same buffer as described above, with a low (10 nM) or a high (1 μM) concentration of (−)-nicotine, respectively.

2.3. D_2 receptor and TH mRNA levels

2.3.1. Oligodeoxynucleotide synthesis and labelling

Following analysis for mRNA secondary structure using GCG Sequence Analysis Software 7.1, oligodeoxynucleotide sequences were chosen in unique regions of the mRNAs and synthesised using a Cyclone (Biosearch) DNA synthesiser. The sequences used were the following:

DA D_2 receptor (recognising both short and long isoforms): 5-C AGG GTT GCT ATG TAG GCC GTG GGA TGG ATC AGG GAG AGT GA-3'[9].

TH: 5'-AGG GTG TGC AGC TCA TCC TGG ACC CCC TCC AAG GAG CGC T[23].

The oligonucleotide probes were labelled at the 3'-end using [^{35}S]dATP (Amersham) and terminal deoxynucleotidyl transferase (Boehringer-Mannheim) following the specifications of the manufacturer to a specific activity of 100–300 KBcq/pmol. The labelled probes were separated from unincorporated [^{35}S]dATP by means of NucTrap push columns (Stratagene, La Jolla), precipitated in ethanol, resuspended in distilled water containing 50 mM dithiothreitol and frozen until use.

2.3.2. In situ hybridization procedure

Brains dissected from rats sacrificed by decapitation were fresh frozen on dry ice. Frozen tissues were cut at the cryostat (14 μm thick sections), thaw mounted on poly-1-lysine coated slides and stored at −80° C for 1–3 days. Sections were fixed with 4% paraformaldehyde for 5 min at room temperature, washed in phosphate buffered saline (PBS) and then acetylated and delipidated in ethanol and chloroform (5 min). They were prehybridized for 4 h at 37° C and hybridized for 20 h at 37° C under parafilm coverslips. The composition of the prehybridization and hybridization mixtures were: 50% formamide, 0.6 M NaCl, 0.1 M dithiothreitol, 10% dextran sulfate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 × Denhardt's solution (50 × = 1% BSA/1% Ficoll/1% polyvinylpyrrolidone), 0.1 mg/ml polyA (Boehringer), 0.5 mg/ml yeast tRNA (Sigma), 0.05 mg/ml herring sperm DNA (Promega) in 0.02 M Tris-HCl, pH 7.5. Probes were applied at a concentration of 2,000–3,000 Bcq/30 μl/section (corresponding to around 15 fmol/section). After removal of coverslips and initial rinse in 2 × standard saline citrate (SSC) solution (3 M NaCl/0.3 M sodium citrate) at room temperature (two times for 5 min each time), sections were washed four times for 15 min each time in 2 × SSC/50% formamide at 42° C and, then two times for 30 min each time in 1 × SSC at room temperature. After rinsing in ice-cold distilled water and drying, they were exposed to Hyperfilm Bmax (Amersham) for 10–20 days.

Autoradiographic films were analysed using the VIDAS image analyzer (Kontron, München, Germany) according to previously published procedures[61]. Briefly, in each section the areas of interest (caudate-putamen and nucleus accumbens in the telencephalic sections, substantia nigra and ventral tegmental area in mesencephalic sections) were interactively selected together with an area where no specific labelling was detected (e.g., the neocortex). The mean grey value was measured in these areas and transformed into specific optical density (SpOD).

2.4. Immunocytochemistry of TH-IR

Transcardiac perfusion was performed under sodium pentobarbital anaesthesia (40 mg/kg, i.p.) 4, 12 or 24 h after (−)-nicotine injection (1 mg/kg, i.p.) with 50 ml saline at 37° C followed by 150 ml cold (4° C) fixative consisting of 0.4% (v/v) picric acid and 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed, postfixed in the same solution for 2 h and rinsed in 10% sucrose/0.1 M sodium phosphate buffer for 3 days at 4° C. Two 14-μm-thick coronal sections were made from the neostriatum (Bregma level −0.4 mm) and substantia nigra (Bregma level −5.8 mm), respectively. These two levels give representative images of the DA nerve terminal in the neostriatum and the DA cells in the substantia nigra. The sections, cut in a Leitz cryostat, were then mounted on slides coated with chrom-alum-gelatin. The sections were incubated for 15 h in a humid chamber at 4° C with a mouse monoclonal TH antibody (Inc-Star, Stillwater, Minn, USA) that recognises an epitope in the midportion of the TH molecule. The TH

antiserum was diluted 1:2,000 in 0.3% Triton X-100 in 0.1 M sodium phosphate buffer. The sections were then incubated in biotinylated anti-mouse antibodies (Vector, Burlingame, CA, USA) diluted 1:200 in 0.1 M PBS for 1 h in darkness at 25° C. The avidin-biotin peroxidase technique (Vectastain, Vector, Burlingame, CA, USA) was employed, using 3,3'-diaminobenzidine (Sigma, St. Louis, USA) as a chromogen.

Densitometric evaluation of the substantia nigra was performed using the IBAS image analyser (Kontron, München, Germany) linked to a CCD 72 camera (Dage, MTI, Michigan city, IN, USA) mounted on a Zeiss microscope with stabilized illumination according to previously published procedures[3,60]. For the analysis of the substantia nigra, a sample field of 0.52×0.56 mm was selected in the medial part of the zona compacta at Bregma level −5.8 mm. For each section, an area with non-specific staining was measured and a discrimination threshold for accepting specifically stained profiles was selected at a fixed distance from the mean value of the non-specific staining. Two parameters of discriminated profiles were obtained: the field area, i.e., the area covered by discriminated profiles, and the SpOD.

In the analysis of the neostriatum, the entire caudate-putamen or nucleus accumbens was measured after acquisition of the section using a low magnification objective of the microscope (Bregma level 1 mm). The regions were manually selected together with an area of non-specific staining and measured. The results were expressed as SpOD values.

In both analyses, four sections per rat were analyzed. In each section the field was bilaterally sampled. The replicates were then averaged for statistical purposes.

2.5. Data analysis and statistical tests

All data from the biochemical binding experiments were analyzed by iterative non-linear regression fitting procedures allowing the use of non-transformed data[41]. Points with standard residuals having an absolute value greater than or equal to two were automatically discarded by the computer. For the saturation experiments the best fit model was a model for one binding site used to determine the K_D (the equilibrium dissociation constant) and the B_{max} (the maximum number of binding sites) values[45].

Only curves, where no more than two out of ten points were removed, were included in the statistical evaluation. The K_D values do not have a normal distribution. To achieve homogeneity of variance and allow parametric statistical analysis, the K_D values were logarithmically transformed[52]. However, the B_{max} values have a normal distribution and therefore need not to be transformed. The statistical significance of the difference between the control and the (−)-nicotine-treated animals in the in vivo and in vitro studies were evaluated with the Student's unpaired and paired t-test (two-tails), respectively. The Jonckheere–Terpstra test was used to analyze the time-related effects of (−)-nicotine on the K_D value of [^{3}H]raclopride binding sites in the in vivo studies. In situ hybridization and immunocytochemical data were analysed by means of one-factor factorial ANOVA followed by the Bonferroni correction procedure for multiple comparisons[58]. The minimum level for statistical significance was set at $P < 0.05$.

3. RESULTS

3.1. Biochemical binding experiments

3.1.1. In vivo effects of (−)-nicotine on D_2 receptors in rat neostriatum

3.1.1.1. Saturation experiments with D_2 antagonist [^{3}H]raclopride. As seen in Table 1, a single injection of (−)-nicotine (1 mg/kg, i.p.) produced a significant reduction in the K_D value of [^{3}H]raclopride binding sites in rat neostriatal membrane preparations at the 12 h time interval. The effects were absent at the 30 min and the 4 h time intervals, and had disappeared after 24 h. In the time interval of 0.5 −12 h after the in vivo treatment of (−)-nicotine, a significant time-re-

Table 1
Saturation experiments with the dopamine D_2 antagonist [^{3}H]raclopride binding in rat neostriatal membranes. Effects of (−)-nicotine (1 mg/kg, i.p.) Rat neostriatal membranes were incubated for 30 min at 25°C. The $K_D$values are presented as antilogarithms (geometric means and 95% confidence limits of the logarithmically transformed data used for statistical analysis), and the B_{max} values are shown as means ± S.E.M, as calculated by non-linear regression from saturation experiments with ten concentrations of [^{3}H]raclopride (1–30 nM) using 100 μM of DA for the determination of non-specific binding. $n = 4$–8 experiments. $^*P < 0.05$ against the respective control group according to the Student's unpaired t-test (two-tails).

Treatment	Time	K_D		B_{max}	
	(h)	(nM)	(%)	(fmol/mg protein)	(%)
Control	0.5	5.28 (4.85–5.75)	100	266 ± 8	100
Nicotine		4.99 (4.51–5.52)	95	300 ± 18	113
Control	4	6.70 (6.08–7.38)	100	240 ± 30	100
Nicotine		5.37 (4.52–6.38)	80	211 ± 34	88
Control	12	7.41 (6.82–8.05)	100	234 ± 18	100
Nicotine		5.15 (4.76–5.57)*	70	217 ± 28	93
Control	24	5.94 (5.33–6.62)	100	279 ± 16	100
Nicotine		4.66 (4.12–5.26)	78	258 ± 35	92
Control	48	6.99 (5.57–8.79)	100	283 ± 35	100
Nicotine		6.76 (5.76–7.92)	97	269 ± 23	95

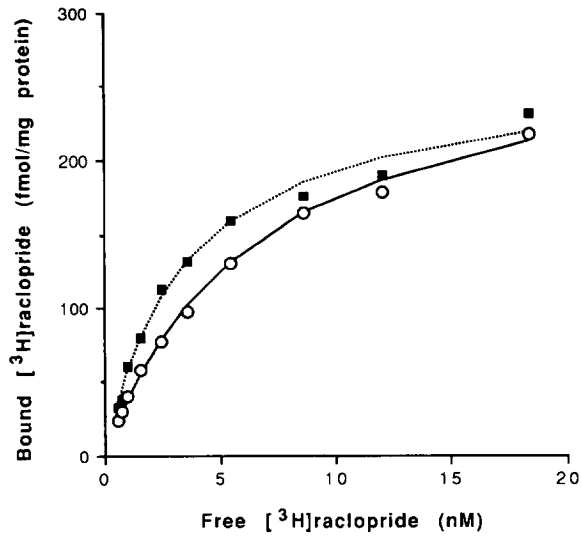

Fig. 1. Representative competition curves illustrating the effects of single dose (1 mg/kg) (−)-nicotine injection (i.p.) on dopamine (DA) D_2 antagonist [^{3}H]raclopride binding in rat neostriatal membranes at the 12 h time interval. Rat neostriatal membranes were incubated for 30 min at 25°C. The K_D and the B_{max} values were 6.63 nM and 253 fmol/mg protein for the control curve (○), and 3.50 nM and 249 fmol/mg protein for the (−)-nicotine-treated curve (■) as calculated by iterative non-linear regression from saturation curves with ten concentrations of [^{3}H]raclopride (1–35 nM). 100 μM of DA was used for the determination of non-specific binding.

lated decrease in the K_D values could be demonstrated by using the Jonckheere–Terpstra test ($P < 0.01$) for ordered alternatives. At no time interval did the in vivo (−)-nicotine treatment result in a change in the B_{max} value. The results at the 12 h time interval are illustrated in Fig. 1. The saturation curve is clearly shifted to the left.

3.1.1.2. Saturation experiments with D_2 agonist [^{3}H]NPA. As seen in Table 2, a single injection of (−)-nicotine (1 mg/kg, i.p.) did not produce significant changes in the K_D and the B_{max} values of [^{3}H]NPA binding sites in rat neostriatal membrane preparations at any time interval tested (0.5–48 h).

3.1.2. Blockade of the (−)-nicotine-induced decrease in the K_D value of [^{3}H]raclopride binding sites by the nicotine antagonist mecamylamine

As seen in Fig. 2, a significant 25% decrease in the K_D value of [^{3}H]raclopride binding sites in the rat neostriatal membrane preparations was found 12 h after the in vivo treatment with (−)-nicotine (1 mg/kg, i.p.). Pretreatment (1 mg/kg, i.p.) with the nicotine receptor antagonist mecamylamine, by itself inactive, completely abolished the decrease in the K_D value induced by (−)-nicotine. Neither (−)-nicotine and mecamylamine alone, nor their combination produced any changes in the B_{max} value (Fig. 2).

3.1.3. In vitro effects of (−)-nicotine on D_2 receptors in rat neostriatum

3.1.3.1. Saturation experiments with the D_2 antagonist [^{3}H]raclopride. As seen in Fig. 3, in vitro, a low (10 nM) or a high (1 μM) concentration of (−)-nicotine failed to alter the characteristics of [^{3}H]raclopride binding in the neostriatal membrane preparations as shown in a saturation analysis.

3.1.3.2. Saturation experiments with the D_2 agonist [^{3}H]NPA. As seen in Table 3, addition of a low (10 nM) or a high (1 μM) concentration of (−)-nicotine to rat neostriatal membrane preparations failed to influence the K_D and the B_{max} values of [^{3}H]NPA binding.

3.2. Effects of (−)-nicotine on D_2 receptor and TH mRNA levels

In agreement with previous reports, high labelling for D_2 receptor mRNA was detected in the neostriatum and the ventral mesencephalon (see Fig. 4, panels A,C). As seen in Table 4, (−)-nicotine at a dose of 1 mg/kg (i.p.) did not induce any significant change in D_2 receptor mRNA levels in any region analysed or at any time interval studied.

TH mRNA labelling was detected in high concentration in the pars compacta of the substantia nigra and ventral tegmental area, and more sparsely in the pars reticulata of the substantia nigra (see Fig. 4, panel E). Computer-assisted densitometry did not show any

Table 2
Saturation experiments with the dopamine D_2 agonist [^{3}H]N-propylnorapomorphine (NPA) binding in rat neostriatal membranes Effects of (−)-nicotine (1 mg/kg, i.p.) Rat neostriatal membranes were incubated for 30 min at 25°C. The $K_D$values are presented as antilogarithms (geometric means and 95% confidence limits of the logarithmically transformed data used for statistical analysis), and the B_{max} values are shown as means ± S.E.M, as calculated by non-linear regression from saturation experiments with ten concentrations of [^{3}H]NPA (0.1–5.0 nM) using 1 μM of raclopride for the determination of non-specific binding. $n = 8$ experiments except for the 24 h treatment with (−)-nicotine ($n = 20$). A Student's unpaired t-test (two-tails) was used.

Treatment	Time	K_D		B_{max}	
	(h)	(nM)	(%)	(fmol/mg protein)	(%)
Control	0.5	0.42 (0.38–0.47)	100	415 ± 15	100
(−)-Nicotine		0.35 (0.34–0.37)	83	410 ± 7	99
Control	4	0.49 (0.43 –0.58)	100	398 ± 12	100
(−)-Nicotine		0.46 (0.43–0.50)	92	344 ± 19	86
Control	12	0.39 (0.37–0.41)	100	456 ± 13	100
(−)-Nicotine		0.36 (0.37–0.38)	92	442 ± 32	97
Control	24	0.44 (0.40–0.46)	100	423 ± 10	100
(−)-Nicotine		0.51 (0.47–0.55)	116	410 ± 6	97
Control	48	0.46 (0.43–0.50)	100	473 ± 19	100
(−)-Nicotine		0.40 (0.34–0.47)	87	491 ± 31	103

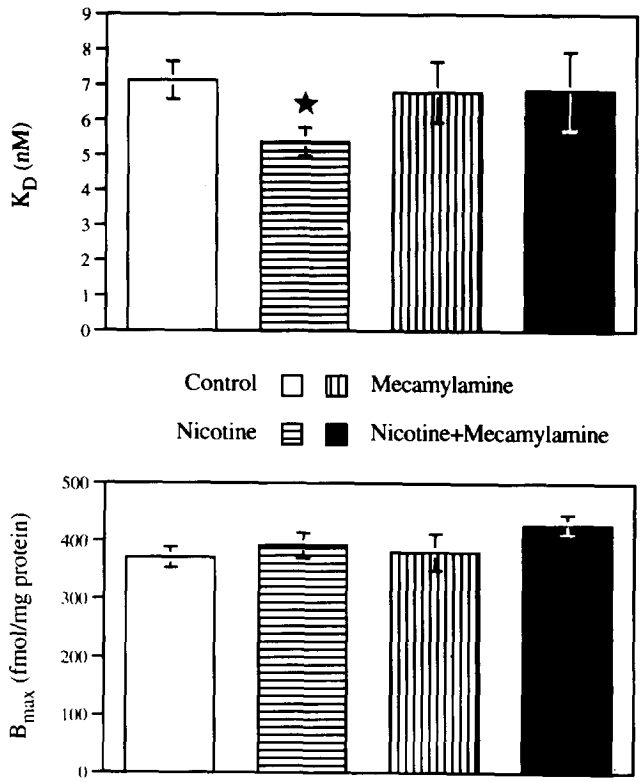

Fig. 2. Influence of the nicotine antagonist mecamylamine (1 mg/kg, i.p. 15 min prior to nicotine injection) on the effects of (−)-nicotine (1 mg/kg, i.p., 12 h before killing) on the K_D and B_{max} values of dopamine D_2 antagonist [^{3}H]raclopride binding sites in rat striatal membrane preparations. Rat neostriatal membranes were incubated for 30 min at 25° C. The K_D and the B_{max} values were 7.13 ± 0.53 nM and 371 ± 18 fmol/mg protein (means ± S.E.M) for the control group as calculated by non-linear regression from saturation experiments with ten concentrations of [^{3}H]raclopride (1–30 nM), using 1 μM of the D_2 antagonist raclopride for the determination of non-specific binding. $^*P < 0.05$ against the control group mean value according to an one-factor factorial ANOVA followed by the Fisher's PLSD test. $n = 8$ rats except for the control group (n = 19) and the (−)-nicotine treated group ($n = 21$).

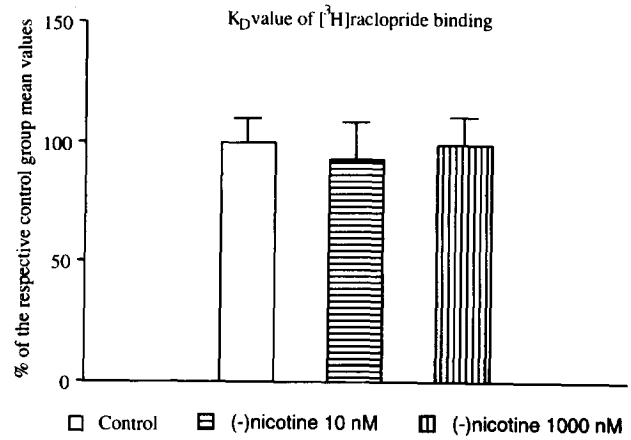

Fig. 3. Effects of (−)-nicotine *in vitro* on the K_D value of the dopamine D_2 antagonist [^{3}H]raclopride binding sites in rat neostriatal membranes. Rat neostriatal membrane preparations were incubated for 30 min at 25° C in the presence or absence of 10 or 1000 nM of (−)-nicotine. The K_D value, presented as means ± S.E.M., was 7.14 ± 0.44 nM for the control group. $n = 8$ experiments except the control group ($n = 33$). No significance was found between the control group and the (−)-nicotine-treated group according to the Student's paired *t*-test (two-tails).

significant effect of (−)-nicotine treatment on TH mRNA levels within the substantia nigra and the ventral tegmental area at any time interval studied (Table 4).

3.3. Effects of (−)-nicotine on TH-IR

High levels of TH-IR were detected in both the neostriatum and the ventral mesencephalon. As seen in Table 5 and Table 6, (−)-nicotine treatment at a dose of 1 mg/kg (i.p.) failed to significantly alter TH-IR at both rostro-caudal levels. However, a trend toward an increase in the TH-IR was observed in neostriatum at the 24 h time interval (Table 6).

4. DISCUSSION

The major finding of the present paper is that a single dose (−)-nicotine injection can increase the affinity of D_2 receptors for the antagonist in the rat neostriatal membrane preparations after a time delay. The increase in the affinity of D_2 receptors for the antagonist [^{3}H]raclopride develops strongly and be-

Table 3
Effects of (−)-nicotine in vitro on the dopamine D_2 agonist [^{3}H]N-propylnorapomorphine (NPA) binding in rat neostriatal membranes. Rat neostriatal membrane preparations were incubated for 30 min at 25°C in the presence or absence of 10 or 1000 nM of (−)-nicotine. The $K_D$values are presented as antilogarithms (geometric means and 95% confidence limits of the logarithmically transformed data used for statistical analysis), and the B_{max} values are shown as means ± S.E.M. $n = 6$ experiments. No significance was found between the control group and the (−)-nicotine-treated group according to the Student's paired *t*-test (two-tails).

Treatment	K_D		B_{max}	
	(nM)	(%)	(fmol/mg protein)	(%)
Control	0.46 (0.41–0.49)	100	414 ± 48	100
(−)-Nicotine 10 nM	0.59 (0.52–0.68)	128	497 ± 66	120
Control	0.50 (0.43–0.57)	100	475 ± 34	100
(−)-Nicotine 1000 nM	0.59 (0.50–0.71)	118	498 ± 41	105

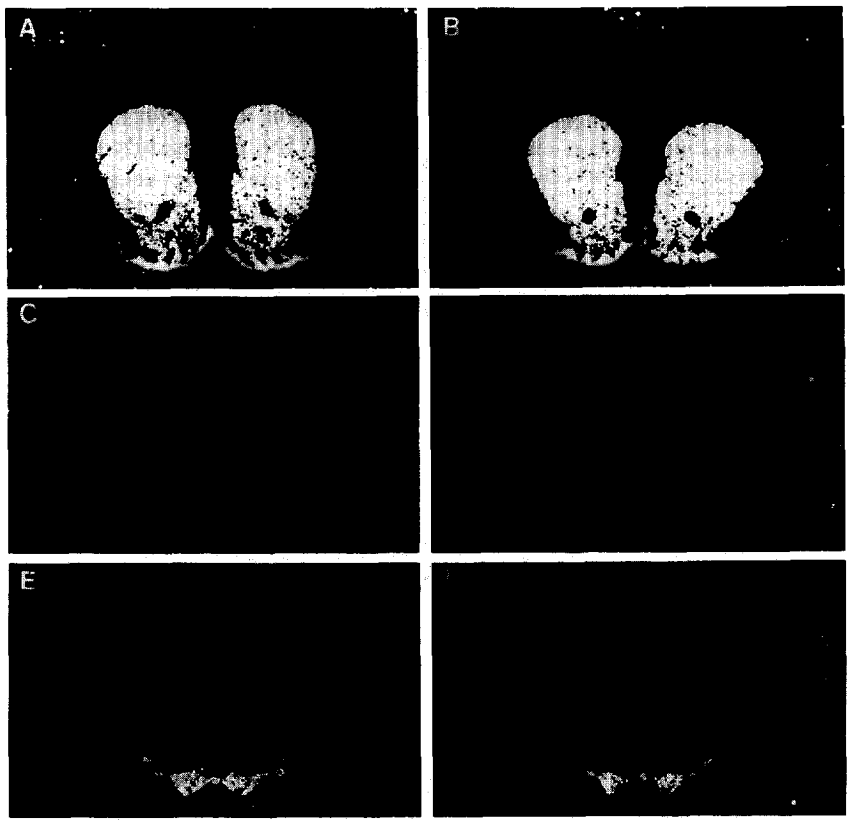

Fig. 4. Autoradiograms showing labelling for dopamine D_2 receptor mRNA (panels A–D) and tyrosine hydroxylase mRNA (panels E, F) in rats 12 h after treatment with saline (panels A, C, E) or with (−)-nicotine (1 mg/kg, i.p., panels B, D, F). Bregma level 1.0 mm for panels A, B, E, F and −5.5 mm for panels C, D.

Table 4
Effects of a single dose (1 mg/kg) (−)-nicotine injection (i.p.) on dopamine D_2 receptor and tyrosine hydroxylase (TH) mRNA levels in rat brain. Specific optical density values are shown as means ± S.E.M. $n = 4–5$ rats for the saline-treated groups, and 6–8 rats for the (−)-nicotine-treated groups. For each rat four densitometric measurements were performed and the values were pooled together for the statistical analysis. One-factor factorial ANOVA followed by the Bonferroni correction procedure for multiple comparisons was used.

Treatment	4 h	12 h	24 h
D_2 receptor mRNA			
Caudate-putamen:			
Saline	0.3608 ± 0.0135	0.3407 ± 0.0177	0.3339 ± 0.0080
(−)-Nicotine	0.3644 ± 0.0093	0.3542 ± 0.0090	0.3232 ± 0.0050
Nuclear accumbens:			
Saline	0.2969 ± 0.0121	0.2566 ± 0.0101	0.2407 ± 0.0074
(−)-Nicotine	0.2765 ± 0.0055	0.2740 ± 0.0177	0.2316 ± 0.0050
Substantia nigra:			
Saline	0.1135 ± 0.0048	0.1210 ± 0.0036	0.1165 ± 0.0060
(−)-Nicotine	0.1061 ± 0.0035	0.1052 ± 0.0033	0.1123 ± 0.0042
Ventral tegmental area:			
Saline	0.0895 ± 0.0042	0.0912 ± 0.0074	0.1165 ± 0.0080
(−)-Nicotine	0.0972 ± 0.0043	0.0820 ± 0.0022	0.1123 ± 0.0067
TH mRNA			
Substantia nigra:			
Saline	0.1407 ± 0.0044	0.1423 ± 0.0041	0.1324 ± 0.0016
(−)-Nicotine	0.1391 ± 0.0040	0.1379 ± 0.0040	0.1336 ± 0.0027
Ventral tegmental area:			
Saline	0.1285 ± 0.0051	0.1246 ± 0.0035	0.1231 ± 0.0055
(−)-Nicotine	0.1281 ± 0.0035	0.1246 ± 0.0023	0.1320 ± 0.0047

Table 5
Effects of a single-dose (1 mg/kg) (−)-nicotine injection (i.p.) on tyrosine hydroxylase (TH) immunoreactivity in rat substantia nigra. The values are shown as means ± S.E.M. $n = 4–6$ rats per group. One-factor factorial ANOVA followed by the Bonferroni correction procedure for multiple comparisons was used. SpArea = area covered by specific profiles, SpOD = optical density of specific profiles.

Treatment	SpArea (μm^2)	SpOD
4 h:		
Saline	37,275 ± 3,789	0.6325 ± 0.0243
(−)-Nicotine	37,920 ± 2,927	0.6396 ± 0.0292
12 h:		
Saline	37,387 ± 3,266	0.6227 ± 0.0120
(−)-Nicotine	35,023 ± 2,699	0.6741 ± 0.0116

comes significant at the 12 h time interval, after which the K_D value slowly returns to normal at the 48 h time interval. The involvement of nicotine receptors in this phenomenon is demonstrated by its blockade by the nicotine antagonist mecamylamine. However, the affinity of D_2 receptors for the agonist [^{3}H]NPA binding sites was not affected by (−)-nicotine at any time-interval studied. These effects are not accompanied by any significant change in D_2 mRNA levels either in intrinsic neostriatal neurones or in mesencephalic dopaminergic neurones projecting to the neostriatum. Thus, the major change caused by a single injection of (−)-nicotine consists in the control of the affinity of the D_2 receptors for the antagonist.

These findings are the first evidence for delayed actions of a single (−)-nicotine injection on neostriatal D_2 receptors. They are of substantial interest, since alterations in mesolimbic DA transmission have been postulated to be involved in producing nicotine dependence[53]. Thus, delayed changes in neostriatal D_2 receptors can take place after an acute dose of (−)-nicotine injection, and the changes may alter D_2 receptor signal transduction It may be speculated that such events if they take place in limbic D_2 receptors may lead to a craving for nicotine to maintain D_2 mediated DA transmission.

At present, it is not known what molecular mechanism(s) may underlie changes in the apparent affinity of D_2 receptors in equilibrium binding experiments. We will briefly discuss two possibilities, i.e., that changes in apparent affinity are due to changes in the proportion of different D_2 isoforms with different affinity for the ligands used, or to regulation of the interconversion between different conformations of a single isoform of the D_2 receptor by covalent modifications.

Table 6
Effects of a single-dose (1 mg/kg) (−)-nicotine injection (i.p.) on tyrosine hydroxylase (TH) immunoreactivity in rat neosriatum. Specific optical density values are shown as means ± S.E.M. $n = 4–6$ rats per group. One-factor factorial ANOVA followed by the Bonferroni correction procedure for multiple comparisons was used.

Treatment	4 h	12 h	24 h
Caudate-putamen:			
Saline	0.1041 ± 0.0102	0.1465 ± 0.0101	0.1189 ± 0.0021
(−)-Nicotine	0.1014 ± 0.0101	0.1297 ± 0.0121	0.1361 ± 0.0068
Nuclear accumbens:			
Saline	0.1006 ± 0.0109	0.1305 ± 0.0043	0.1016 ± 0.0060
(−)-Nicotine	0.0989 ± 0.0098	0.1178 ± 0.0076	0.1176 ± 0.0076

Regarding the first hypothesis, a family of molecules showing D_2-like pharmacology has been cloned, including short and long D_2 isoforms, the D_3 and the D_4 isoforms. Interestingly, D_2 and D_3 isoforms have a lower affinity for apomorphine than the D_4 isoform, but have a higher affinity for raclopride[20]. A shift in the proportion of D_2/D_3 vs D_4 isoforms could thus explain changes in D_2 apparent binding affinity. However, available data make this possibility unlikely. In fact, while D_2 isoforms are highly expressed in the nigrostriatal pathway, D_3 and D_4 isoforms have not been detected at appreciable levels in these regions[20]. Moreover, we have found that D_2 mRNA levels are unchanged upon a single (−)-nicotine injection in both neostriatum and ventral mesencephalon.

Regarding the second hypothesis, it has been shown that different allosteric conformations of ligand-gated ion channels display different relative affinities for agonists and antagonists. For instance, the desensitised state of the nicotinic acetylcholine receptor has a high affinity for agonists but low affinity for antagonists, whereas the resting state has a high affinity for antagonists but low affinity for agonists[10]. Reversible allosteric modulation[33], such as receptor phosphorylation[24], can shift the equilibrium between resting and desensitised states.

In the case of G-protein-linked receptors, like the D_2 family of receptors, phosphorylation can change the rate of short-term desensitisation[13,24,43]. Receptor phosphorylation can be mediated by receptor-specific kinases (such as b-ARK) and binding of arrestin-like molecules (homologous phosphorylation, i.e., due to DAergic agonist binding to the receptor) as well as by non-specific protein kinases such as protein kinase A (heterologous phosphorylation, i.e., due to activation of other receptors). In both cases, phosphorylation results in G-protein uncoupling, which in turn causes the stabilisation of a conformation of the receptor with decreased affinity for the ligand[47]. Interestingly, it has been shown that activation of protein kinase C can regulate the affinity of D_2 receptors[46]. Thus, changes in the phosphorylation state of D_2 receptors may explain changes in apparent affinity of the receptor in equilibrium binding experiments.

Several mechanisms may be considered for how nicotine might elicit the observed changes in D_2 receptor affinity. Nicotine effects might be mediated by the activation of nigrostriatal DAergic neurones (see intro-

duction). Accordingly, the observed nicotine effects would be analogous to those of other brain stimulants, namely amphetamine, cocaine and bupropion, which increase neostriatal DA availability[54]. It has been shown that single cocaine, amphetamine or bupropion (as well as other stimulants) treatments increase in vivo D_2 antagonist binding in neostriatum[26,54], a result compatible with present data on [^{3}H]raclopride binding. However, in vitro data are more contradictory[38] and are not easy to compare with the present results. Alternatively, the changes in D_2 receptors might be mediated by nicotine/DA receptor interactions[17,62]. Though present results on the absence of in vitro modulation of D_2 binding by nicotine makes a direct intramembrane receptor–receptor interaction unlikely, an indirect modulation through phosphorylation remains a distinct possibility. It has, in fact, been shown that neuronal-type nicotinic receptors are highly permeable to calcium ions[42,51,55]. Calcium entry would then activate protein kinases such as calcium/calmodulin-dependent protein kinases and protein kinase C leading to changes in the phosphorylation state (and, possibly, in long-lasting changes in their transcriptional levels) of neostriatal proteins regulating D_2 receptors or of the receptors themselves (see above).

Finally, nicotine may modulate D_2 receptors through the intervention of neostriatal neuropeptides. It has been previously demonstrated that cholecystokinin (CCK) octapeptide can increase the affinity of D_2 receptors for the antagonist[1,34] whereas neurotensin/neuromedin N can decrease the affinity of D_2 receptors for the agonist[2,34,36] in rat neostriatum. It may, therefore, be speculated that nicotine causes prolonged increase in CCK peptide release responsible for the increase in D_2 antagonist binding affinity.

That at least part of nicotine effects on D_2 receptors is mediated by DA release remains an intriguing possibility. Other mechanisms, such as prolonged changes in neostriatal neuropeptide release, may also be at work (see above). Further studies directly investigating these possibilities will help clarify the issue.

At variance with what has been recently observed in the locus coeruleus where a single injection of (−)-nicotine causes a delayed several-fold increase in TH mRNA[40], no effects of single injection of (−)-nicotine on TH mRNA levels were observed in the substantia nigra and ventral tegmental area at the various time intervals studied. In agreement with the present results, Mitchell et al.[40] have also reported the failure to increase TH mRNA levels in the ventral midbrain. Consistently, no significant change in TH-IR was detected either in mesencephalic cell bodies and neuropil or in neostriatal nerve terminals at the time interval studied.

In conclusion, the present findings demonstrate that a single injection of (−)-nicotine produces delayed changes in the affinity of neostriatal D_2 receptors for the antagonist but not for the agonist. Furthermore, the D_2 receptor mRNA or TH mRNA and protein levels in the mesostriatal pathway have not been changed by the in vivo treatment with (−)-nicotine.

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