Full Paper

Long-Term Effects of High Doses of Nicotine on Feeding Behavior and Brain Nitric Oxide Synthase Activity in Female Mice

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Abstract. We studied the long-term effects of repeated doses of nicotine, causing dependence, 120 days after its withdrawal on feeding behavior and on brain nitric oxide (NO) formation in female mice. Nicotine dependence was induced by subcutaneous (s.c.) nicotine injection (2 mg/kg, four injections daily) for 14 days. Daily food intake was evaluated for the entire observational period (120 days). Moreover, 30, 60, and 120 days after nicotine withdrawal, we evaluated food intake, nitrite/nitrate levels, and nitric oxide synthase (NOS) activity and expression in the hypothalamus after food deprivation (24 h). In animals in which nicotine dependence was induced (NM), daily food intake was similar to that of controls (M). However, following food deprivation, NM mice showed i) a significant increase in food intake, ii) changes in weight gain and in hypothalamic nitrite/nitrate levels, and iii) enhancement of hypothalamic neuronal NOS (nNOS) activity. Results indicate that high doses of nicotine producing dependence induce long-term changes in feeding behavior consequent to food deprivation associated to alterations in the brain nitrergic system.

Keywords: food intake, nicotine, nitric oxide, weight gain, food deprivation

Introduction

Nicotine is considered responsible for most of the effects on health consequent to the smoking habit. An interesting aspect related to smoking is the link between tobacco use, food intake, and body weight gain. It is well known that smoking cessation causes an increase in food consumption and body weight gain (1), while weight of habitual smokers is lesser than non-smokers (2). An increase in body weight is considered, especially among women, an obstacle to definitive smoking cessation. Experiments on female rats have shown that nicotine administration reduces food intake and body weight, while its withdrawal induces the opposite (3). Most studies investigating nicotine effects have generally reported results obtained by experiments conducted during nicotine exposure or only for short periods after discontinuing nicotine use. In addition, the studies published on the relationship between nicotine and food intake (4) are generally time-limited. For this reason we investigated the long-term effects of repeated administration of high doses of nicotine on feeding behavior, 120 consecutive days after withdrawal.

Nitric oxide (NO), synthesized from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS), acts as a neurotransmitter and plays a modulatory role on food intake (4, 5). Interactions between nicotine and NO in the regulation of food intake have not been sufficiently investigated. However, it has been observed that peripheral chronic injection of nicotine increases, especially in female rodents, the level of NO metabolites (6).

In the light of this, we studied in female mice the long-term (120 days) after effects of nicotine withdrawal: feeding behavior, brain NO metabolites (nitrite /nitrate), and NOS activity in the hypothalamus.

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Materials and Methods

Female, Swiss 8-week-old mice, weighing $25 - 30$ g, housed at a constant temperature ($22 \pm 2°C$) and a light cycle of $12/12$ h (7.00 a.m. / 7.00 p.m.) with free access to standard chow pellets and drinking water, unless otherwise stated, were used. Animals were randomly allocated in different groups, and one week before the start of the experiment, baseline body weight and food intake were measured. Adaptation and experiments were carried out in accordance with the internationally accepted principles and the national laws concerning the care and the use of laboratory animals and with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

To induce nicotine dependence, the mice were injected with saline (controls, M) or nicotine hydrogen tartrate salt (Sigma-Aldrich, Milan, Italy) (NM) at 2 mg /kg, s.c., respectively, four injections daily, 4 h apart, starting at 08:00 h for 14 days. This intermittent nicotine treatment more closely resembles human use (7).

Locomotor activity was evaluated in all the groups 24 h after the last injection of saline or nicotine in an open field apparatus subdivided into nine communicating squares. The animals were placed in the apparatus and the number of squares crossed were determined in 6 min (8). Locomotor activity was evaluated by observers that were blind to the drug treatment.

Abstinence signs were evaluated by four experienced observers kept unaware of the drug treatment at 24 h into withdrawal. Prior to each observation session, N and NM were placed in clear cages to habituate for 30 min and then were returned after 60 min (adaptation: 30 min, observation: 30 min) to their home cage upon completion of the behavioral evaluation. For the evaluation of the behavioral signs, a Nicotine Abstinence Scale was used (7). The Nicotine Abstinence Scale scored the frequency of the following signs: rearing, jumping, body shakes, head shakes, forelimb shakes, scratching, chewing, abdominal constrictions, and facial tremor during 30 min of observation. The severity of the abstinence syndrome was evaluated by computing the total abstinence sign score (7, 9).

Food intake and body weight gain were measured for 120 consecutively days, starting from nicotine withdrawal, every 24 h at 10:00 a.m. Food intake was measured by an automated computerized "eater meter" developed in our laboratory for these studies. Data of food intake were expressed as grams per 24 h per mouse.

At 30, 60, and 120 days after nicotine withdrawal, a group of animals were deprived of food but not of water. After 24 h, food was presented and consumption was evaluated for two successive hours. At the same times (30, 60, and 120 days from nicotine withdrawal), a group of animals was killed 24 h after food deprivation under light ether anesthesia to measure nitrite and nitrate and NOS activity in the hypothalamus. The brains were rapidly removed and the hypothalamus was dissected out and frozen at $-70°C$. $NO_2 + NO_3$ levels were measured with the Griess reagent, as previously described (10) with some modifications. Briefly, aliquots ($250 \, \mu l$) of supernatant previously homogenized (1:10, w/v) in phosphate buffer (0.1 M, pH 7.4) were diluted with ultrapure water ($500 \, \mu l$; Seromed Biochrom, Berlin, Germany) and incubated at room temperature with $250 \, \mu l$ substrate buffer (0.1 M imidazole, $210 \, \mu M$ NADPH, $3.8 \, \mu M$ flavine adenine dinucleotide, pH 7.6) in the presence of nitrate-reductase (*Aspergillus niger*, 10 UI/l; Sigma-Aldrich) for 45 min to convert NO_3 to NO_2. Total NO_2 ($NO_2 + NO_3$) was analyzed by reacting the samples with Griess reagent (58 mM sulfanilamide and 3.8 mM naphtalene-ethylene diamine dihydrochloride in 0.5 M H_3PO_4). Reacted samples were treated with $200 \, \mu l$ trichloroacetic acid (1.2 M) and centrifuged for 5 min at $8000 \times g$, and adsorbance of the supernatant was measured at 540 nm (Microspectrophotometer mod. 340 ATTC; SLT Lab. Instruments, Salzburg, Austria). Amounts of NO_2 were estimated from a standard curve for $NaNO_2$ obtained by enzymatic conversion of $NaNO_3$ ($0 - 32 \, \mu M$). Data were expressed as μM per mouse.

To measure neuronal NOS (nNOS) activity in the same experimental conditions (24-h food deprivation), brain tissues were removed from food-deprived animals and homogenized at $4°C$ in 4 vol of HEPES buffer (20 mM, pH 7.2) containing 320 mM sucrose, 1 mM DL-DTT, and $10 \, \mu g/ml$ leupeptin. The homogenates were centrifuged at $100,000 \times g$ for 30 min at $4°C$. The supernatants were stored at $-70°C$ until use. Protein concentration in the cytosolic fractions was measured spectrophotometrically according to Bradford's method, using BSA as standard (11).

Activity of NOS was evaluated by measuring the rate of conversion of L-[U-14C]arginine to [U-14C]citrulline according to the method of Salter et al. (12) and was expressed as nanomoles of citrulline per minute per gram of tissue. Briefly, an aliquot of cytosolic fraction (containing 100 mg of protein) was preincubated for 5 min at $37°C$ in 50 mM potassium phosphate buffer (pH 7.2) containing 60 mM L-valine, $120 \, \mu M$ NADPH, 1.2 mM L-citrulline, 1.2 mM $MgCl_2$, and 0.24 mM $CaCl_2$, in the presence of drug or vehicle. Samples were then incubated for 10 min at $37°C$ with L-[U-14C]arginine (150,000 dpm) and $20 \, \mu M$ L-arginine. The reaction was stopped by the addiction of 1.0 ml of a 1:1 vol/vol mixture of H_2O and Dowex-50W (8% cross-linked

Na$^+$ form, Dry MESH 200-400). The Na$^+$ form of Dowex-50W was prepared by washing the H$^+$ form of the resin 4 times with 1 M NaOH and then with double-distilled water until the pH was less than 7.5. The resin was settled by centrifugation at $11,000 \times g$ for 3 min in a Beckman Microfuge 11 (Beckman Coulter Inc., Fullerton, CA, USA), and an aliquot of the supernatant was used for scintillation counting with 4 ml Pico-Flour (Packard Instrument Co., Meriden, CT, USA). The activity of Ca^{2+}-dependent nNOS was determined from the difference between the [U-^{14}C]citrulline produced by control samples and that produced by samples containing 1 mM EGTA. Activity of the Ca^{2+}-independent enzyme was determined from the difference between the [U-^{14}C]citrulline produced by samples containing 1 mM EGTA and that produced by samples containing 1 mM EGTA and 1 mM L-N^G-monomethyl arginine. Data were expressed as nmol/min per gram.

For Western blot analysis, animals were sacrificed on the 15th day of nicotine or saline treatment (last day) and at 15, 30, 45, and 60 days after withdrawal. The diencephali were homogenized in 1 ml of 62 mM Tris-HCl (pH 6.8), 5% SDS, 1 mM aprotinine, 10 mM 2-mercaptoethanol, and 10% glycerol. The homogenates were centrifuged at $15,000 \times g$ for 10 min at 4°C, and the supernatants were collected. Protein concentration was determined by the DC Protein Assay (Biorad, Milan, Italy). Samples (50 μg of protein) were run on 7.5% PAGE and transferred electrophoretically onto a polyvinile membrane.

The membrane was incubated with rabbit anti-nNOS, polyclonal antibody (1:500 dilution, Catalog n AB1552; Chemicon International, Milan, Italy) in TBS 0.1% Tween 20 buffer at 4°C over night. After rinsing with TBS 0.15% Tween 20, the membrane was incubated in 5% dried milk, with horse anti-rabbit peroxidase conjugate secondary antibody (1:8000 dilution) for 1 h at room temperature. After the final rinse, the immunoreactive bands in the membrane were visualized using ECL Western blot detection reagents (Amersham Bioscience).

All statistical procedures were performed using SPSS statistical software package release 6.1.3. (SPSS, Chicago, IL, USA). Data analysis was performed using two-ways analysis of variance (ANOVA) with the Scheffè post-hoc test for multiple comparisons. Each group of animals was composed of six mice. The data are expressed as the means ± S.E.M. Statistical significance was set at $P<0.05$.

Results

Discontinuing the intermittent administration of nicotine caused a reduction in locomotor activity. M
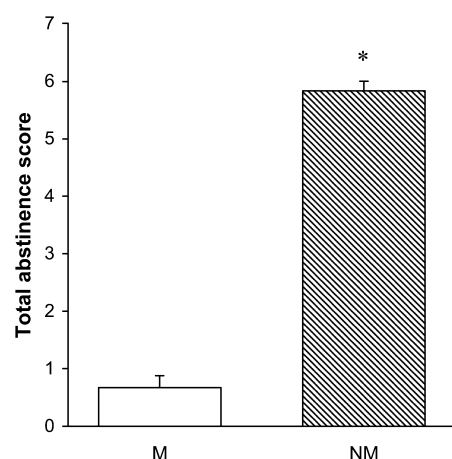

Fig. 1. Total abstinence score during nicotine withdrawal after 14 days of saline (M) or nicotine (NM; 2 mg/kg, four injections daily, s.c.). Each column represents the mean ± S.E.M. of six animals. *$P<0.01$ vs M.

crossed a mean (± S.E.M.) of 72.7 ± 3.28 squares. NM crossed 39.5 ± 3.4 squares ($P<0.001$). The total abstinence signs score was significantly higher in NM (score: 5.83 ± 0.17) compared to M (score: 0.67 ± 0.21) (Fig. 1), thus indicating the development of dependence.

Daily food intake evaluated during the entire observation period (120 days) following nicotine withdrawal did not show changes between M and NM animals. The M animals ate 4.95 ± 0.15 g/mouse and NM animals ate 4.98 ± 0.09 g/mouse with small and not significant changes during the whole period of observation. Measurement of body weight detected weekly during the observation period (120 days) showed light and not significant differences in the early period of withdrawal between M and NM (first week of withdrawal: 28 ± 1.2 and 30 ± 1.3 g, respectively), but did not show any significant difference between the two groups for the rest of the observation period. Evaluation of 2-h food intake after 24 h of food deprivation performed at 30, 60, and 120 days from nicotine withdrawal showed a significant increase in NM with respect to M. Food intake after 24-h food deprivation in M animals was 0.47 ± 0.03, 0.50 ± 0.02, and 0.55 ± 0.02 at 30, 60, and 120 days, respectively. Food intake after 24-h food deprivation in NM animals was 0.62 ± 0.03, 0.68 ± 0.03, and 0.73 ± 0.03 at 30, 60, and 120 days, respectively (Fig. 2). Nitrite and nitrate levels (index of NO synthesis) measured in the hypothalamus in the same range of time were significantly increased in the group of NM compared to M (Fig. 3). Finally, activity of hypothalamic nNOS, found to be increased in 24-h food-deprived animals, was further enhanced in NM. Only Ca^{2+}-dependent NOS activity representing constitutive

nNOS was detected. This activity was significantly increased in all food-deprived animals compared to the not deprived animals. This increase was significantly
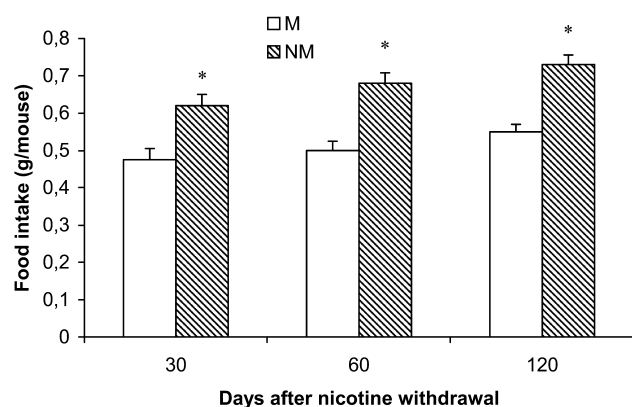
larger in NM deprived in comparison to M deprived animals (Table 1), Western blot analysis showed that hypothalamic nNOS expression decreased significantly in NM compared to M, even after food deprivation, for the entire observation period (Fig. 4).

Fig. 2. Two hours food intake after 24-h food deprivation performed in animals 30, 60, and 120 days from nicotine withdrawal. Each column represents the mean ± S.E.M. of six animals. *P<0.01 vs M.
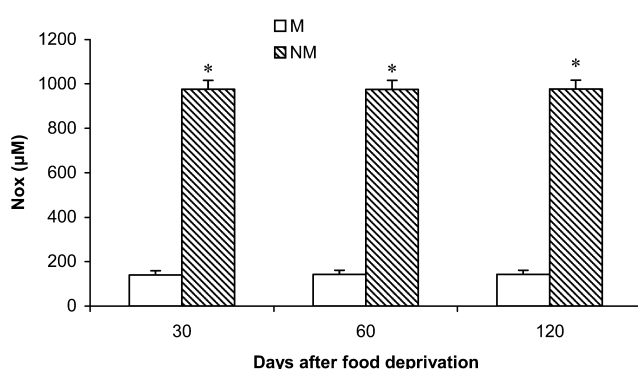

Fig. 3. Measurement of nitrite/nitrate levels after food deprivation (24 h) in the hypotalamus of mice previously treated for 14 consecutive days with saline (M) or nicotine (NM; 2 mg/kg, four injections daily, s.c.), performed at 30, 60, and 120 days after nicotine withdrawal. Each column represents the mean ± S.E.M. of six animals. *P<0.01 vs M.

Discussion

It is thought that nicotine reduces appetite and increases energy intake, while in subjects that quit smoking, these effects are lacking and they show hyperphagia and enhancement of body weight gain (13). Women that stop smoking often gain more weight than men (14) and frequently they continue smoking to control their weight (15).

It has been shown that hypophagia induced by nicotine is associated with increased serotonin and dopamine levels in the hypothalamus, whereas hyperphagia, after nicotine cessation, was accompanied by a decreased concentration of these neurotransmitters, thus suggesting that nicotine affects appetite regulation in part by modulating serotonin and dopamine in the hypothalamus (16). The importance of the hypothalamus in the regulation of metabolism and feeding behavior has been well documented (17). We investigated the long term-effects of nicotine on NO formation in the hypothalamus, another neurotransmitter involved in ingestive behavior.

NO is a versatile molecule (18, 19): it acts as modulator of synaptic transmission, is released from smooth muscle cells of blood vessels, and inhibits platelet aggregation (20). Inhibition of NO formation consequent to administration of NOS activity inhibitors reduces food ingestion in the laboratory animal (21), and it has also been shown that food deprivation causes an increase in NOS activity (22). NO is also involved in hypophagia induced by leptin (23, 24), and levels of the enzyme NOS in the hypothalamus are increased by the orexigenic compound ghrelin (25).

Table 1. Measurement of hypothalamic neuronal NOS activity after food deprivation in mice previously treated for 14 consecutive days with saline (M) or nicotine (2 mg/kg, four injections daily) (NM), at 30, 60, and 120 days from nicotine withdrawal

Animals	NOS activity (nmol citrulline/min per gram)		
Days	30	60	120
M	0.95 ± 0.03	0.96 ± 0.03	0.95 ± 0.03
M + food deprivation	1.42 ± 0.06*	1.43 ± 0.07*	1.43 ± 0.06*
NM	0.98 ± 0.04	0.99 ± 0.04	0.98 ± 0.03
NM + food deprivation	1.84 ± 0.07°,#	1.84 ± 0.06°,#	1.85 ± 0.07°,#

Each value represents the mean ± S.E.M. of six animals. *P<0.001 vs M; °P<0.001 vs NM; #P<0.001 vs NM + food deprivation.
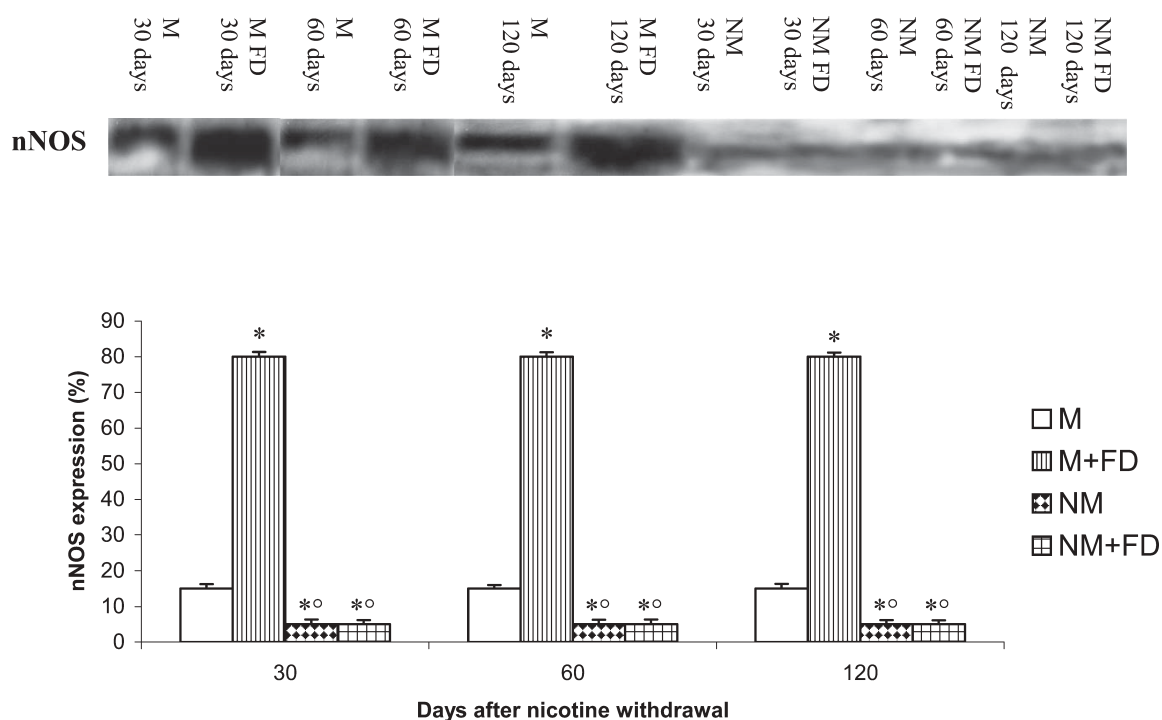
Fig. 4. Hypothalamic nNOS expression measured with Western blot analysis 30, 60, and 120 days after nicotine withdrawal, 24 h after food deprivation, in M and NM food deprived (FD) animals. Percentages are expressed as arbitrary units of integrated intensity. Each column represent the mean ± S.E.M. of six animals. *$P<0.01$ *vs* M; °$P<0.01$ *vs* M + FD.

Nicotine influences NO synthesis and is released into the central nervous system (26), and moreover, in rats chronically exposed to cigarette smoke, a decrease in nNOS activity has been found (6). More recently, it was also reported that nicotine administration decreases NOS expression in the hypothalamus of food-deprived rats (4).

In the present work, we investigated the long-term changes in feeding behavior observed in animals previously exposed to a nicotine regimen in doses producing dependence. In addition to daily food intake, body weight gain detected weekly in the months following nicotine withdrawal also show light but not significant modifications compared to animals that never received nicotine. In particular, mice that received nicotine (NM) in normal conditions ate (when food is always daily available) an amount of food comparable to the controls (M), and body weight gain of NM animals was comparable to that of M animals for the entire 120 days. Weight gain after stopping smoking is commonly cited, especially among women. A light body weight gain is observable also in our experiments in animals previously exposed to nicotine. The difference in weight gain compared to untreated animals is not significant and is similar to the lower percentage of weight gain (about 6 – 7%) observed in humans after stopping smoking

(27). However, in the experiments performed at different times after nicotine withdrawal, in which animals were food-deprived, when food was presented, NM ate larger amounts of food in the first 2 h of presentation compared to M, thus indicating that fasting produces a different behavior in animals previously exposed to nicotine. This effect was evident at 30 days after nicotine withdrawal and still present at 120 days after withdrawal.

Nicotine influences food intake and body weight by modulating the levels of several neurotransmitters (4). One of these compounds could be NO. Interactions between feeding behavior and NO have been investigated, and it has been shown that food deprivation caused a marked increase in nNOS activity (22). Our experiments showed that in animals pre-exposed to nicotine and food deprivation, this increase is significantly greater than in the controls. It has been shown that acute or chronic nicotine administration increases the stable metabolites of NO (28). Nicotine seems to influence the release of NO through elevations of intracellular Ca^{2+}. In particular, nicotine promoting Ca^{2+} entry would stimulate NOS into the cells that contain the enzyme and would result in enhanced NO generation (29, 30). The present work seems to confirm these data in light of the finding that NO activity and metabolites are increased in animals pre-exposed to

nicotine and indicate that this could be a long-term effect. Our experiments also show that expression of neuronal hypothalamic NOS is reduced in animals pre-exposed to nicotine. Previously, it was shown that nicotine acute administration reduces nNOS expression in the hypothalamus of food-deprived rats (4). Also, in our experiments, it is evident that nicotine administration reduces hypothalamic nNOS expression, but indicate that after chronic nicotine administration, this effect can last long and is not dependent on food deprivation.

The role of NO in the central regulation of food intake has been related to the neurotransmitter serotonin, and it has been hypothesized that an increase in brain NO following food deprivation could be linked to a decrease in brain serotonin (22). Thus, it is possible to think that previous exposure to high doses of nicotine, by influencing NO metabolism, could cause a long-term disturbance of NO release and consequently of serotonin modulation and eating behavior. In light of this hypothesis, alteration in brain NO formation could be responsible for the different eating behavior observed in mice pre-exposed to nicotine. In conclusion, our results show that in animals pre-exposed to high doses of nicotine producing dependence, alterations in feeding behavior are present for a long time (120 days) after nicotine withdrawal. These changes are characterized by increased food intake after food deprivation, and they are probably related to alterations in L-arginine – NO pathway. Considering that four months of life in a rodent are comparable to a considerable part of human life, these results suggest that similar alterations could be present in humans for some time after quitting smoking.

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