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Chronic nicotine treatment decreases neurofilament immunoreactivity in the rat ventral tegmental area

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Abstract

Region-specific decreases of neurofilament proteins have been described in the ventral tegmental area of rats chronically treated with either morphine or cocaine. The aim of the present study was to assess if the levels of neurofilament proteins are changed in the ventral tegmental area by chronic treatment with nicotine. Immunoreactivity for NF-68, NF-160 and NF-200 was determined using NR4, BF10 and RT97 antibodies, respectively. Measurements were performed using computer-assisted microdensitometry of brain sections from rats exposed to chronic nicotine treatment (0.4 mg/kg/day × 6 days) or to saline. Chronic nicotine treatment reduced NF-160 and NF-200 immunoreactivity by 44.5% ($P < 0.01$) and 22.5% ($P < 0.05$), respectively, in the ventral tegmental area but not in the substantia nigra. A trend towards reduction was observed for NF-68 immunoreactivity in the ventral tegmental area. These preliminary results suggest that nicotine shares the same properties with cocaine and morphine to reduce neurofilament proteins in the ventral tegmental area, a key brain structure of the reward system.

Keywords: Nicotine; Neurofilament; NF-68; NF-160; NF-200; Ventral tegmental area; Immunohistochemistry

1. Introduction

Chronic addictive drug exposure induces adaptive changes in neural circuits that ultimately modify the function of emotional and motivational processes involved in addictive behavior. In recent years, several authors have shown that particular biochemical substrates (transcription factors, immediate early genes, receptors, G proteins, and kinases) are altered by chronic treatment with either cocaine or morphine at doses that are known to produce changes in rat behavioral outcome, such as locomotor activation, preference for drug solutions, and drug self-administration (Nestler et al., 1993). The neuroanatomical sites specifically affected by chronic cocaine or morphine treatment include the ventral tegmental area and the nucleus accumbens (Nestler et al., 1993), the origin and the main terminal field of the mesolimbic dopamine system, respectively (LeMoal and Simon, 1991). A vast array of data has implicated this system in the reinforcing effects of drugs of abuse (Koob, 1992; Kreek and Koob, 1998).

Among the various biochemical changes, reduction in the expression of three of the neural intermediate filament proteins of the type IV family, NF-68, NF-160 and NF-200 (Zuoshang et al., 1994), was described for the ventral tegmental area of rats exposed to chronic treatment with morphine, alcohol or cocaine (Beitner-Johnson et al., 1992; Nestler et al., 1994). Parallel biochemical and behavioral studies on biochemical changes in the ventral tegmental area of inbred Lewis and Fischer rat strains have shown that, phenotypically, the former strain resembles the latter on exposure to chronic treatment with morphine, cocaine or alcohol (Nestler et al., 1993). Moreover, naive Lewis rats showed reduced neurofilament protein levels in the ventral tegmental area similar to those described after chronic cocaine or morphine treatment (Guitart et al., 1992, 1993b), supporting the hypothesis that changes in neurofilament proteins are involved in the genetically determined 'drug-preferred' state.

These findings suggest that chronic use of drugs of abuse generates long-term changes in the expression of proteins involved in the maintenance of the neuronal cytoskeleton. These changes eventually participate in the neuroadaptation of the activity of the mesolimbic dopaminergic pathway that is believed to act as substrate for

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addictive behavior (Guitart et al., 1993a; Miserendino et al., 1993).

Nicotine, similar to other drugs of abuse, activates the mesolimbic pathway by increasing cell firing of dopamin-

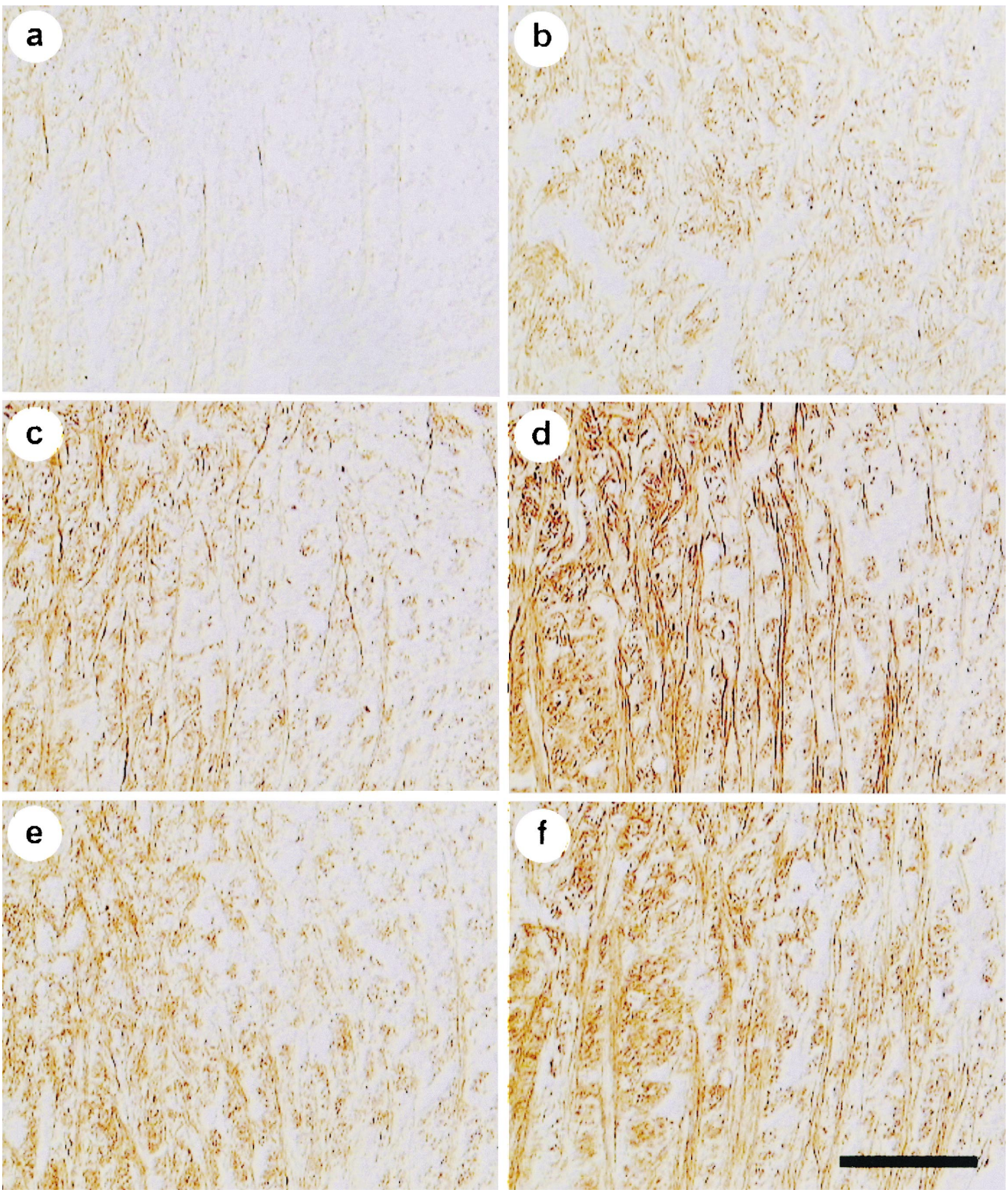

Fig. 1. Microphotographs of the distribution of neurofilament proteins immunoreactivity in the ventral tegmental area of the rat. NF-68 immunoreactivity is represented in panels (a) and (b); NF-160 immunoreactivity is represented in panels (c) and (d); NF-200 immunoreactivity is represented in panels (e) and (f). The effects of chronic nicotine treatment (0.4 mg/kg/day s.c. for 6 days) are visible in panels (a), (c), and (d) and are compared with those of saline treatment, represented in panels (b), (d) and (f). Calibration bar: 50 μm.

ergic neurons in the ventral tegmental area through activation of nicotinic receptors (Calabresi et al., 1989; McGehee et al., 1995). Interestingly, chronic nicotine treatment results in sensitization of dopamine release in response to nicotine microinfusions into the ventral tegmental area (Balfour et al., 1998) in a manner that is reminiscent of the effects of psychostimulants.

The aim of the present study was to investigate the changes of neurofilament proteins in the ventral tegmental area following chronic nicotine treatment according to regimens similar to those known to produce sensitization of dopamine release to challenging doses of nicotine (Balfour et al., 1998). Validated computer-assisted morphologic methods were applied to measure changes in immunoreactivity for NF-68, NF-160, and NF-200 neurofilament proteins in regions of the ventral tegmental area and substantia nigra that contain dopaminergic neurons.

2. Material and methods

2.1. Subjects and drug treatments

Male Wistar rats (initial body weight 170–240 g; Charles River, Calco, Italy) were used. Animals were housed in three or four per cage and maintained in a temperature-controlled environment on a 12-h light–12-h dark cycle with light on at 6:00 a.m. with food pellets and water ad libitum. All animal procedures and research complied with national legislation and with the company policy on the Care of Use of Animals and with related codes of practice.

Chronic nicotine treatment consisted of daily subcutaneous nicotine injections (0.4 mg/kg expressed as free base; Sigma) between 9:00 and 10:00 a.m. every day for 6 consecutive days. The control rats received subcutaneous saline injections (1 ml/kg) under the same conditions. Six animals per group were included in the experiment.

2.2. Immunohistochemistry

The rats were killed under urethane anesthesia by intraventricular perfusion with ice-cold phosphate buffered saline (PBS) followed by ice-cold 4% paraformaldehyde in PBS for 25 min (10 ml/min). The brains were carefully removed and maintained in the fixative overnight. The samples were then kept in PBS up to paraffin embedding. Sections containing the ventral tegmental area and substantia nigra were cut at 4 μm thickness according to the atlas of Paxinos and Watson (1986) using a Microm rotative microtome. The following mouse monoclonal antibodies (NovoCastra Labs.) and dilutions were used in this study: NR4 to detect NF-68 (1:20); BF10 to detect NF-160 (1:20); RT97 to detect NF-200 (1:100). The sections were dewaxed and antigen retrieval was performed by microwave treatment with three cycles of 5 min at 800 W, followed by a single cycle of 10 min at 600 W for NF-68 and NF-160 in citrate buffer (pH 6) and one cycle of 5 min at 800 W for NF-200 in citrate buffer (pH 6). The slides were then incubated with hydrogen peroxide in methanol to quench endogenous peroxidase activity. Antibodies were applied and incubated overnight at 4°C. After a wash in PBS, the sections were incubated with an avidin–biotin complex (LSAB 2, Dako) and developed using 3-3′ diaminobenzidine tetrahydrochloride as chromogen.

2.3. Image analysis

Six identical-size sample fields (ventral tegmental area, substantia nigra, and corpus callosum, on both the left and right hemisphere) were digitized from each section, using a Nikon Microphot FXA Light Microscope at a magnification of 200×. Measurements were performed on three different sections per animal, and the average was used for statistical analysis. The neurofilament protein immunreactive area was measured using the MCID image analysis

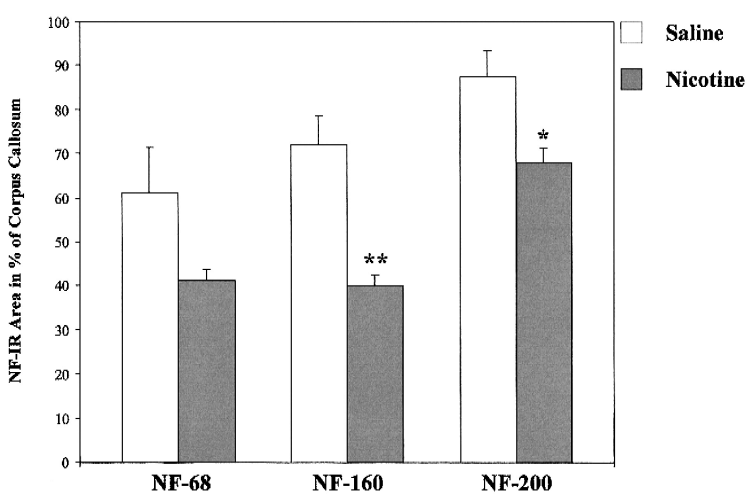

Fig. 2. Changes in neurofilament immunoreactivity (NF-IR) area in the ventral tegmental area of rats after chronic nicotine (0.4 mg/kg/day s.c. for 6 days) or saline. Values are expressed as means ± S.E.M. percentage of NF-IR area in corpus callosum. $^{*}P < 0.05$ and $^{**}P < 0.01$, one-way ANOVA; $n = 6$ rats.

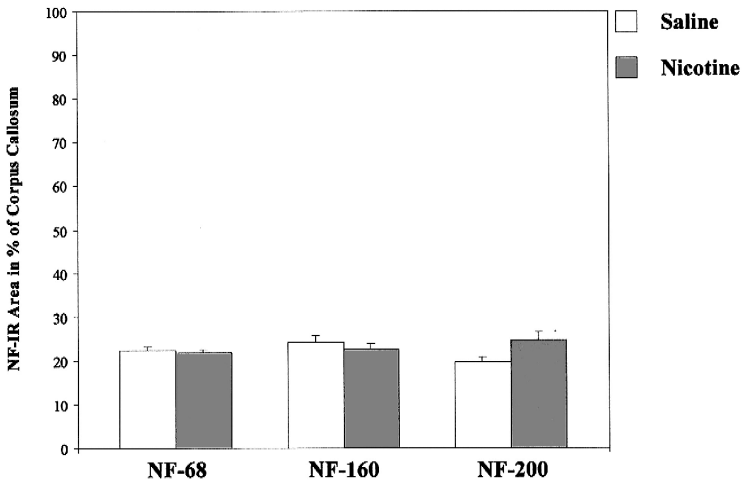

Fig. 3. Changes in neurofilament immunoreactivity (NF-IR) area in the substantia nigra of rats after chronic nicotine (0.4 mg/kg/day s.c. for 6 days) or saline. Values are expressed as means ± S.E.M. percentage of NF-IR area in corpus callosum. $^{*}P < 0.05$ and $^{**}P < 0.01$, one-way ANOVA; $n = 6$ rats.

software (Imaging Research). Sample field images were processed with an enhancement filter and subsequently discriminated by applying a gray level threshold, assuring that the immunoreactive area measured represented exclusively neurofilament protein immunoreactivity. Area values were normalized towards the corpus callosum values, and subsequently analyzed using one-way analysis of variance (ANOVA).

2.4. Cell count

Cresyl violet staining was performed to visualize neurons in the ventral tegmental area from sections adjacent to those used for immunohistochemistry. Sections of 4 μm from paraffin embedded rat brains were dewaxed in xylene and rehydrated with decreasing concentrations of ethanol. After 1–2 min of staining in Cresyl fast violet, sections were differentiated in 95% ethanol containing 10% acetic acid and a few drops of chloroform, dehydrated in ascending concentrations of ethanol and mounted in entellan. Cell count was performed visually on three microscopic fields per section, on two sections per animal, and on three animals per treatment group using 20× magnification (Axiophot, Zeiss, Germany). Results were expressed as means ± S.D.

3. Results

Visual inspection suggested that immunoreactivity for all neurofilament proteins was reduced in the ventral tegmental area of rats exposed to chronic nicotine when compared to that of rats exposed to saline (Fig. 1a–f). Statistical analysis of the microdensitometric measurements of ventral tegmental areas indicated that the reduction in immunoreactivity was significant for NF-160 ($P < 0.01$) and NF-200 ($P < 0.05$), showing a relative reduction of 44.5% and 22.5%, respectively, when compared to that in saline-treated rats (Fig. 2). A non-significant trend towards reduction (32.9%) was obtained for NS-68 immunoreactivity in the ventral tegmental area. No difference was observed when immunoreactivity measurements in the substantia nigra were compared for any of the neurofilament proteins under study (Fig. 3). The cell count in the ventral tegmental area showed no difference between groups (saline: 58.8 ± 2.64 cells/field; nicotine: 60.75 ± 2.63 cells/field).

4. Discussion

The data presented in this article demonstrate that exposure to chronic nicotine treatment reduced neurofilament protein immunoreactivity in the ventral tegmental area of Wistar rats. The reduction did not depend on loss of neurons, since the cell count showed no difference between experimental groups. The observed reduction of NF-160 and NF-200 immunoreactivity and the trend to a reduction observed for NF-68 immunoreactivity could be interpreted as the result of negative transcriptional regulation triggered by the chronic nicotine treatment. Interestingly, expression of neurofilament proteins is greatly enhanced following the neural terminal differentiation that occurs during late post-natal development, and is absent from the brain during fetal and early embryonic life (Escurat et al., 1990). It is tempting to envision the reduced expression of neurofilament proteins produced by nicotine in adult animals as one of the effects of the reactivation of transcriptional regulatory mechanisms normally active during embryonic development and repressed in the adult brain. According to this view, the transcriptional control typical of embryonic life is now recruited to face the neuroadaptation required by chronic drug exposure.

Alternatively, post-translational regulation of the neurofilament proteins may have occurred, i.e., changes in the phosphorylation state that could lead to different organization of neurofilaments in the cytoskeleton, partially mask-

ing the recognition sites for the monoclonal antibodies. Since an apparent increase in the phosphorylation state of NF-160 and NF-200 is reported with chronic cocaine and morphine treatment (Beitner-Johnson et al., 1992) and heavy phosphorylation is generally associated with stability of the neurofilament cytoskeletal organization (Hisanaga et al., 1994), it is difficult to reconcile this interpretation with the observations. Interestingly, chronic cocaine or morphine treatment also increases the phosphorylation of α-internexin, another neurofilament protein member of the IV family and involved in cystoskeletal stability, pointing to a complex regulation produced by drugs of addiction.

Neurofilament proteins are important for determining the axon diameter of neurons that send their projections to distant targets (Zuoshang et al., 1994), as the dopamine neurons of the ventral tegmental area do. Nestler et al. (1993) suggested that chronic exposure to drugs of addiction produces a reduction of the axon diameter of the mesolimbic dopamine neurons, resulting in impaired control of several neural functions, in particular axonal transport to distal synapses. Interestingly, levels and activity of tyrosine hydroxylase, the rate-limiting synthesis enzyme for dopamine, were found increased in the ventral tegmental area following chronic cocaine or morphine treatment (Beitner-Johnson et al., 1992) of drug-preferring rat strains (Miserendino et al., 1993), and after chronic nicotine treatment (Smith et al., 1991). This evidence suggests either positive transcriptional regulation or reduced axonal transport of tyrosine hydroxylase towards the terminal fields.

In conclusion, chronic nicotine-induced effects on neurofilaments are similar to the effects already described for chronic cocaine, ethanol and morphine, giving further evidence for a common neural substrate for the reinforcing properties of drugs of addiction that involves the mesolimbic dopamine system. Electrophysiological, biochemical and morphological studies have indeed confirmed these similarities (Altman et al., 1996; Merlo Pich et al., 1997; Kreek and Koob, 1998), suggesting that common mechanisms underlie the almost identical drug-seeking and drug-taking behavior observed in rodents. Based on these similarities, it can be speculated that chronic exposure to nicotine, as in regular smokers, also may induce long-lasting biochemical modifications that could be reflected in the maintenance and/or relapse of nicotine self-administration, i.e., smoking behavior.

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